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13. ABSTRACT (Maximum 200 Words)

The overall aim of the research is to develop a better understanding of the role of MUC1 in breast cancer. Loss of Muc1 (mouse homologue of MUC1) significantly reduces tumor progression in polyomavirus middle T antigen (PyV MT)-induced mammary tumors. The high transforming activity of the PyV MT antigen depends on c-Src which has been shown to phosphorylate the cytoplasmic tail of MUC1. Our aim is to identify specific proteins that associate with MUC1 and induce signaling that potentiates tumorigenesis, specifically the modulation of c-Src activity and signaling in MMTV-PyV MT tumorigenesis. We have found that MUC1 and c-Src interact in PyV MT-induced mammary tumors. Our data provide insights into the possible mechanism for the significant delay in tumor progression that is observed in the absence of Muc1. We suggest that the interaction of Muc1 with c-Src, a key player in PyV MT transformation, promotes the binding of c-Src to its downstream targets and influences its intracellular localization.

Other studies in the lab have shown that overexpressed MUC1 induces mammary gland tumors. We found that overexpressed MUC1 also inhibits mammary gland involution. These results suggest that MUC1 functions as a weak oncogene in the mammary gland.

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INTRODUCTION

MUC1 (MUC1 in humans, Muc1 in mice) is the founding member of a large and evolutionarily conserved family of proteins known as mucins [1, 2]. Mucins are broadly defined as proteins with extensive O-linked glycosylation, which constitutes 50% of the total molecular mass. MUC1 is a transmembrane mucin with a large, heavily glycosylated extracellular domain and a highly conserved cytoplasmic tail [2]. It is normally expressed on the apical surface of most simple secretory epithelia and several hematopoietic cell lineages [2]. MUC1 was initially cloned from mammary carcinomas as a tumor antigen [3]. More than 90% of human breast cancers overexpress aberrantly glycosylated MUC1 [4]. In cancer cells, the pattern of MUC1 expression is altered such that it is no longer restricted to the apical surfaces, instead it is expressed intracellularly and around the entire cell surface [5]. While MUC1 has been recognized as an important tumor antigen, the identification of a functional role for that overexpression has been elusive. Transgenic and knockout mice have provided insight into the role of MUC1 in tumor development, with the loss of Muc1 resulting in a reduction in tumor progression. Transgenic mice expressing the polyomavirus middle T antigen (PyV MT) under control of the mouse mammary tumor virus (MMTV) promoter exhibit a significant delay in tumor growth and a trend towards decreased metastasis when bred onto a Muc1 knockout background [6]. Also, lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 model of breast cancer [7]. Mammary tumors of MMTV-PyV MT and MMTV-Wnt-1 transgenic mice overexpress Muc1, and as such recapitulate the overexpression of human MUC1 observed in most breast carcinomas. Together, these studies indicate that MUC1 is not merely overexpressed in breast cancer, but also plays an important role in the progression towards invasive carcinoma. Accumulating evidence suggests a functional role for MUC1 in signal transduction. The 72 amino acid tail of MUC1 contains 7 tyrosines, six of which are 100% conserved across mammalian species [8]. The cytoplasmic tail of MUC1 is phosphorylated on tyrosine residues in epithelial cell lines and mouse mammary gland and contains potential docking sites for SH2 containing proteins in addition to a number of possible kinase recognition sites [8-11]. Indeed, MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as EGFR, erbB2, erbB3, erbB4, c-Src, PKCδ, Grb2, β-catenin, GSK3β, and p120^{ctn} [11-17].

We investigated the mechanisms underlying the delay in tumor progression in the absence of Muc1 in the MMTV-PyV MT mouse model of breast cancer in an effort to dissect the signaling role of Muc1 and its contribution to transformation. In the MMTV-PyV MT mammary tumors, activation of the intracellular kinase c-Src is pivotal to the PyV MT-induced transformation [18]. Following c-Src binding and activation, PyV MT modulates the activities of a variety of cellular proteins that function as regulators of cell proliferation such as protein phosphatase 2A, phosphatidylinositol 3-kinase (PI3K), Shc, 14-3-3 proteins and phospholipase C-γ1 [19]. As mentioned above, c-Src is among the signaling molecules known to associate *in vitro* with the cytoplasmic tail of MUC1. c-Src phosphorylates the MUC1 cytoplasmic domain at the YEKV sequence and is involved in regulating the interaction of MUC1 with GSK3β and β-catenin [12]. We hypothesize that MUC1, through its interaction with c-Src, is an important contributor to PyV MT-induced transformation and functions as a scaffold for oncogenic kinases and signaling proteins. Our data suggest that Muc1 functions to promote PyV MT signaling mediated by c-Src activation.

In addition to determining the effect of Muc1 expression on oncogenic signaling in the MMTV-PyV MT model, we examined the effect of MUC1 overexpression in the mammary gland. We observed that the overexpression of human MUC1 in the mouse mammry gland driven by the MMTV promoter resulted in frank neoplasia accompanied by a failure of multiparous glands to dedifferentiate and involute. We have determined that this lack of involution can be observed in non-tumor bearing mammary glands as well, in that post-lactational involution is delayed by MUC1 overexpression. These findings shed new light on the role of MUC1 in the mammary gland, suggesting that it functions as a weak oncogene.

BODY

To understand the mechanism by which Muc1 affects PyV MT-induced tumorigenesis, we examined the effect of Muc1 expression on c-Src. A detailed study of the effect of Muc1 on c-Src signaling in PyV MT-induced tumors was accepted for publication in the journal Oncogene (2005) May 9; [Epub ahead of print]. The publication entitled "Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis" is appended in the report. The above-mentioned publication will be referred to as Manuscript 1.

Virgin female transgenic mice that express the PyV MT oncogene, driven by the MMTV promoter, develop multifocal mammary tumors by 2 months of age and by 4 months greater that 50% of the mice develop lung metastases [20]. The intracellular tyrosine kinase, c-Src, plays a pivotal role in mediating the oncogenic effects of the PyV MT antigen. As described in Table 1, the mouse models used in this study include MMTV-PyV MT transgenic mice either on a Muc1$^{+/+}$ wildtype background (MT) or a Muc1$^{-/-}$ knockout background (MTK). A detailed description of the Muc1$^{-/-}$ knockout mice was previously published [6]. We also crossed the MMTV-PyV MT mice with mice expressing the human MUC1 transgene to generate MMT bitransgenics. The MUC1 transgenic mice express the entire human MUC1 gene sequence including the 1.5 5kb upstream of the MUC1 gene to drive its expression [21]. Normal mammary glands from age-matched wildtype mice were used as controls. Figure 1 in Manuscript 1 shows a marked increase in the level of c-Src expression in the MT and MMT tumors compared with normal mammary glands. Interestingly, the expression of other c-Src like kinases was evident only in the tumor samples.

As described in the May 2003 report, we have established that Muc1 physically interacts and colocalizes with c-Src in MT and MTK tumors by co-immunoprecipitation and immunofluorescence studies (Figures 1 and 2). This falls in line with previous *in vitro* studies that described the interaction between Muc1 and c-Src and thus substantiates a physiological relevance for the association.

Previous findings have demonstrated that lack of Muc1 expression in mice causes a significant delay in PyV MT-induced mammary tumorigenesis. In the PyV MT tumors, we observed an interaction between Muc1 and a key molecule in PyV MT transformation, c-Src. We next investigated the effect of the Muc1/c-Src interaction on PyV MT-mediated transformation. We proposed two mechanisms by which Muc1 could promote tumorigenesis. One is that Muc1 may directly influence c-Src kinase activity and promote c-Src activation by inducing conformational changes or recruiting c-Src activating molecules. Alternatively, Muc1 could influence c-Src signaling by promoting its association with downstream signaling molecules.

We investigated the effect of Muc1 expression on c-Src kinase activity. c-Src was immunoprecipitated from lysates of MT, MMT, MTK tumors and normal wildtype mammary

glands. Kinase assays were performed to determine c-Src activity. The assays were performed using a Src kinase assay kit from Upstate Biotechnology (Lake Placid, NY). The assay measures the amount of $^{32}\gamma$ P-ATP incorporated into a c-Src substrate peptide. The rational behind those experiments was to determine whether the interaction between Muc1 and c-Src was allowing c-Src to assume a more active state possibly through a change in c-Src conformation or through the indirect association of Src with an activating kinase or phosphatase mediated by binding to the cytoplasmic tail. As expected we observed elevates Src kinase activity in the MMTV-PyV MT tumors compared to wild-type mammary glands. In order to address the question of whether Muc1 modulates c-Src kinase activity, we compared c-Src activity in the MT tumors with the MTK tumors lacking Muc1. As reported previously, preliminary results suggested that tumors lacking Muc1 (MTK) exhibited reduced c-Src kinase activity when compared with MT tumors that express Muc1. However, analysis of increased mouse numbers did not substantiate these results. At that point we had analyzed full-grown tumors from MT and MTK animals. We thought that the presence of Muc1 plays a role at the initial stages of tumorigenesis; hence we might see a greater difference in c-Src kinase activity between MT and MTK glands at an earlier time point. To this end, we have harvested and analyzed mammary glands from both MT and MTK mice at 8 weeks of age, at which point the mammary glands would be hyperplastic. We did not observe a significant difference in c-Src kinase activity between the MT and MTK hyperplastic glands (Figure 3). This result is perhaps not surprising, given previous results that showed that the associated kinase activities in mammary tumors decoupled from either the Shc or the PI3K signaling pathway were comparable to that of wildtype PyV MT [22]. The failure to observe a significant difference in c-Src activity in the absence of Muc1 could be attributed to the heterogeneity of the tumor samples and the elevated Src kinase activity in the tumors, whereby subtle changes in c-Src activity could pass undetected. These observations suggest that the delayed tumorigenesis was not a consequence of the inability to functionally activate c-Src tyrosine kinase.

In the absence of a global effect for Muc1 on c-Src kinase activity, we directed our efforts towards analysis of c-Src downstream signaling pathways in the presence or absence of Muc1. Among the c-Src substrates that we investigated is β-catenin. β-catenin is a component of the E-cadherin mediated cell-cell junctions. The tyrosine phosphorylation of β-catenin by c-Src has been shown to inhibit the binding of β-catenin to E-cadherin and disrupt cell-cell adhesions and promote cancer cell invasion [23]. We investigated the association between c-Src and β-catenin in the MT and MTK hyperplastic glands. MT and MTK samples were immunoprecipitated with an anti-c-Src antibody and blotted with an anti-β-catenin antibody. An increased association between c-Src and β-catenin was observed in the presence of Muc1 in the MT samples compared with the MTK samples that lack Muc1 (Figure 4). Based on previously published *in vitro* findings, MUC1 is thought to compete with E-cadherin for β-catenin binding and is therefore involved in disrupting cell-adhesion complexes [15]. β-catenin interacts with Muc1 at an SXXXXXSSL site present in the cytoplasmic tail of MUC1 and that this interaction is enhanced by the tyrsoine phosphorylation of MUC1 cytoplasmic tail by c-Src [15]. Since c-Src has been shown to interact with Muc1 and β-catenin in PyV MT-induced tumors, we were interested in determining whether Muc1 expression would influence the association between β-catenin and E-cadherin. To address that question we immunoprecipitated β-catenin from MT, MMT and MTK tumors and immunoblotted for E-cadherin to assay for β-catenin/E-cadherin association. According to the model proposed by Kufe *et al.* we expected to see a decrease in β-catenin/E-cadherin association in the MT tumors that have overexpression of Muc1 compared with MTK tumors that lack Muc1. However, analysis of MT and MTK tumors did not reveal a substantial change in the amount of β-catenin in association with E-cadherin (Figure 5), suggesting an alternate mechanism for weakening cadherin-dependent adhesions in PyV MT tumors.

We also observed increased levels of activated pStat3, a downstream target of c-Src activation, in the presence of Muc1 (Figure 6). Stat3 is involved in promoting cell-cycle progression, cellular transformation and preventing apoptosis [24-27]. Some PyV MT tumors that lack Muc1 exhibit a decrease in Erk2 activation (*data not shown*). Activation of MAPK in association with MUC1 has been demonstrated *in vitro* and in the mammary glands of MMTV-MUC1 transgenic mice. [11, 28]. Increases in Stat3 and Erk2 phosphorylation could provide a mechanism for increased progression of Muc1-expressing PyV MT tumors. Muc1 expression may be enhancing the activation of Stat3 and Erk2, possibly by its association with EGFR and c-Src. Interestingly, analysis of MT and MTK hyperplastic glands suggested a decrease in EGFR tyrosine phosphorylation in MTK glands lacking Muc1 (*data not shown*).

The p85 subunit of phosphatidylinositol 3-kinase (PI3K) is among the key signaling molecules that associate with the PyV MT antigen [29, 30]. Activation of PI3K signaling influences cell proliferation, growth, survival and cytoskeletal organization [31-33]. We investigated whether the presence of Muc1 influenced the interaction of Src with p85. The interaction between c-Src and the p85 subunit of PI3K kinase was evident only in the MT hyperplastic glands that express Muc1 but not in the MTK glands that lack Muc1 (Figure 7). In a previously published study, transgenic mice expressing a mutated form of PyV MT decoupled from p85 binding develop mammary epithelial hyperplasias that are highly apoptotic, suggesting that PyV MT binding to p85 affects cell survival [22]. The analysis of MT and MTK glands did not reveal changes in the activation of Akt, a downstream target of PI3K signaling affecting cell growth and cell survival. Also, MT and MTK hyperplasias and tumors did not exhibit a difference in the rate of apoptosis, suggesting that this may not be the major pathway affected by the lack of Muc1 expression (unpublished data). In addition to influencing growth and survival, PI3K activation mediates activation of the Rac GTP binding protein, which plays a major role in remodeling the actin cytoskeleton [34]. The p85 subunit of (PI3K) is also among the well-characterized c-Src interacting partners. The association between c-Src and the p85 subunit of PI3K is thought to modulate the localization of activated c-Src at the cell membrane [35]. As such, the presence of Muc1 may be influencing the localization of c-Src at the cell membrane in PyV MT-induced tumors.

The alterations in the association of Src with its downstream effector molecules suggested a possible difference in the cellular localization of Src. In order to address that question we generated primary cell cultures from the mammary epithelial cells derived from MMTV-PyV MT glands on a wildtype or Muc1 null background. In the Muc1 null cells, c-Src is more commonly localized to the perinuclear region as opposed to being localized at the cell membrane in close proximity to its downstream targets (Figure 8B). This suggests that Muc1 is directly or indirectly influencing c-Src localization at the cell membrane. We demonstrate a novel association between Muc1 and a known Src substrate, FAK, in the PyV MT tumors (Figure 8A), which potentially promotes the peripheral targeting of activated c-Src. FAK is as a nonreceptor protein-tyrosine kinase localized within focal adhesions where the cytoskeleton interacts with proteins of the extracellular matrix via integrin receptors. FAK plays an important role in integrin-mediated signaling and modulating processes such as cell growth, differentiation, survival and migration [36]. Following activation by c-Src phosphorylation, FAK interacts with a number of downstream signaling molecules including the adapter protein Grb2 and the p85 subunit of PI3K [37]. In malignant cells MUC1 expression is no longer restricted to apical surfaces but is found throughout the entire cell surface. The binding of MUC1 to Src may help localize the kinase at the cell periphery where it can be in close proximity to target molecules such as β-catenin, FAK and p85. The interaction between MUC1 and FAK has not been described previously. Further investigation into the regulation of this association will shed light on the role of MUC1 on invasion and metastases in the context of integrin-mediated cell-matrix interactions. Figure 10 represents a model for the possible ways Muc1 could influence c-Src

signaling. As such the ability of Muc1 to potentiate c-Src signaling could influence the proliferation and migration of tumor cells. Our understanding of the critical role of Muc1 signaling in PyV MT-induced oncogenesis will be invaluable in delineating the effects of MUC1 expression in human breast cancer.

We had proposed to utilize phage display to identify novel proteins that interact with MUC1 cytoplasmic domain. The MUC1 cytoplasmic tail peptides were used as baits to screen a breast tumor cDNA T7 phage library (Novagen). We carried out several rounds of screening. However we were faced with the problem of non-specific binding and we failed to obtain true positive interactions. We tried several strategies to reduce the non-specific binding first by changing the immobilization solid from 96 well plates to streptavidin beads then by switching to a more stringent panning protocol. Phage display is known to have a high false positive rate. These studies were not pursued further due to the high false positive rates and novel findings discussed below.

While addressing the question of Muc1 signaling in the PyV MT induced tumors, we observed that transgenic mice overexpressing human MUC1 transgene developed mammary tumors. A detailed study of the MMTV-MUC1 tumors and involuting mammary glands was published in the journal . The publication entitled "MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation" is appended in the report and is referred to as Manusript 2.

MMTV-MUC1 MMTV-MUC1 transgenic mice from two different founders (#9 and #15) were continuously bred for twelve months and then monitored as they aged. Cytoplasmic domain-deleted MUC1 transgenic mice (MMTV-MUC1ΔCT, #23) and wildtype controls were housed similarly. Tumors were observed in five out of eight MMTV-MUC1 mice and none were observed in the eight age-matched wildtype mice. As such tumor incidence in the MMTV-MUC1 transgenic mice was 62% higher than in the wildtype mice (p=0.03, 95% CI=0.17 to 0.91). Statistical significance was determined using the Fisher Exact Test. Pathological analysis of the tumors indicated a wide range of tumor subtypes in the MMTV-MUC1 transgenics including papillary carcinoma, solid carcinoma, adenosquamous carcinoma, microacinar carcinoma and lobular hyperplasia (Figure 1 in Manuscript 2). While primary mammary gland tumors were observed in both of the MMTV-MUC1 lines, no tumors were observed in either the MMTV-MUC1ΔCT transgenic line (3 mice sacrificed and analyzed at 8 months and 3 mice at 20 months of age) or the wildtype control lines (analyzed at 20 months). Molecular analysis of these tumors shows a tumor-specific co-immunoprecipitation between MUC1 with β-catenin (Figure 4 in Manuscript 2). Examination of the contralateral glands in MMTV-MUC1 transgenics demonstrates that the development of frank carcinomas is accompanied by a failure of multiparous glands to undergo post-lactational involution (Figure 2 in Manuscript 2). Furthermore, uniparous MMTV-MUC1 transgenic mice display decreased postlactational apoptosis, elevated WAP expression and aberrant pErk2 activation (Figures 6-10 in Manuscript 2). These findings are the first to determine that MUC1 overexpression promotes in vivo transformation of the mammary gland.

MT   MMTV-PyV MT/Muc1$^{+/+}$

MTK   MMTV-PyV MT/Muc1$^{-/-}$

MMT   MMTV-PyV MT/MUC1$^{+/0}$
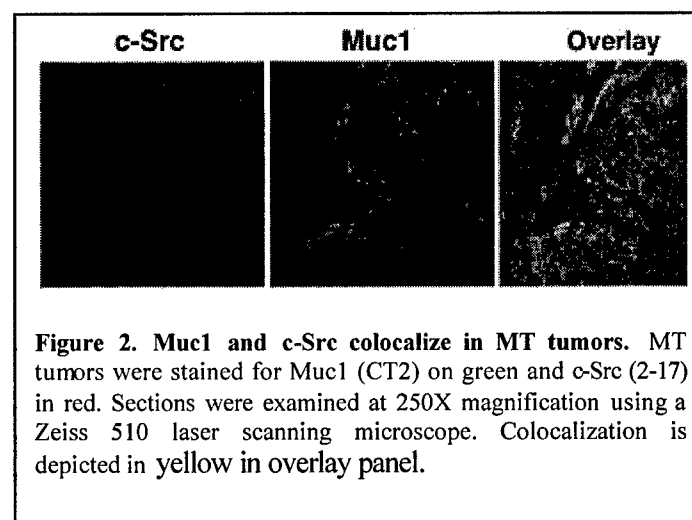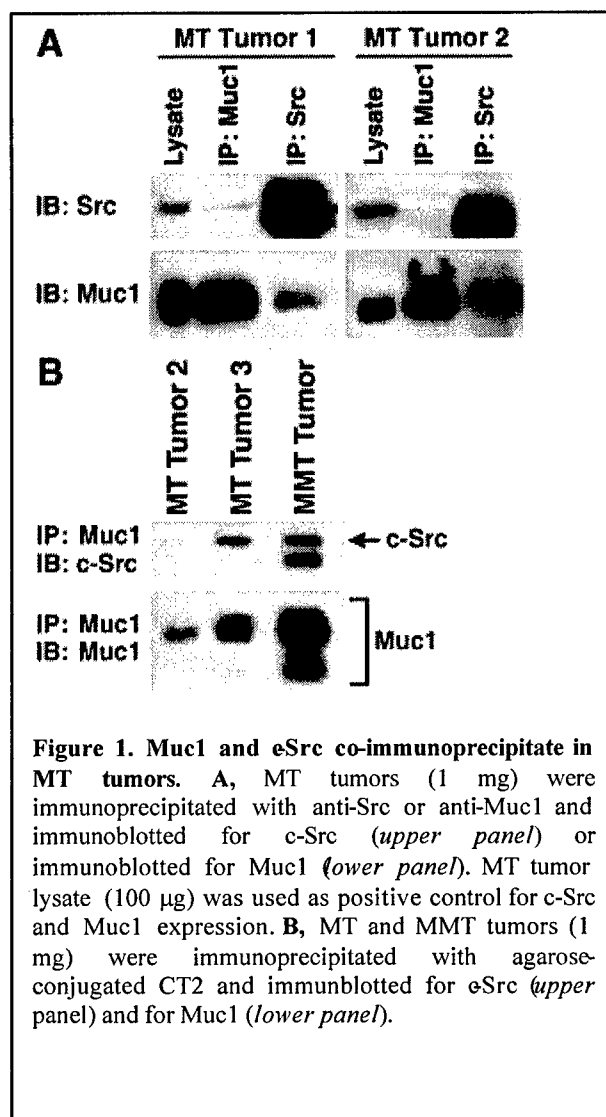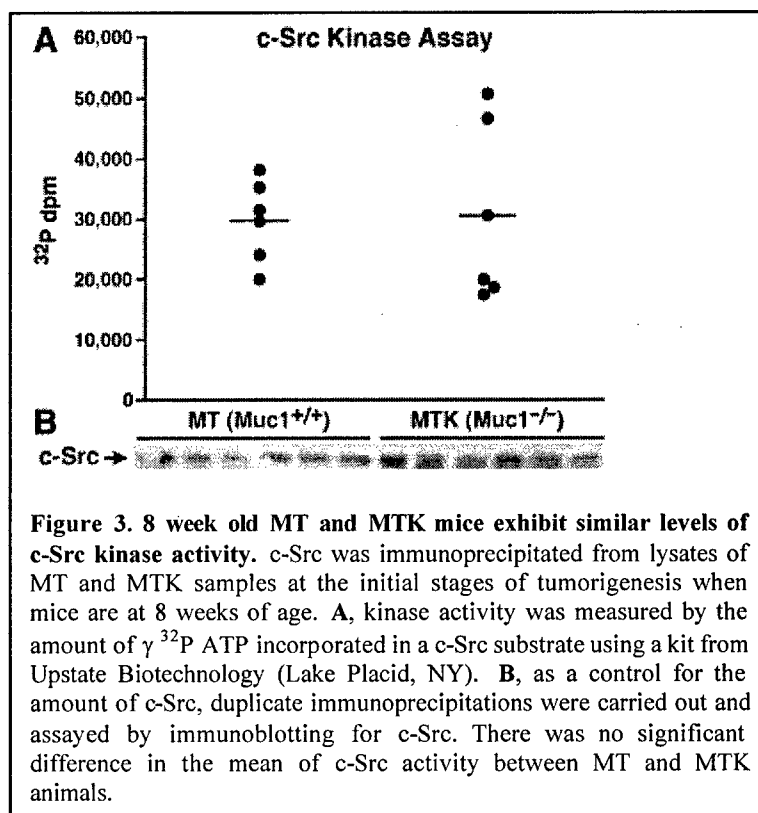
Table 1. List and genotype of mouse models used.

Figure 1. Muc1 and cSrc co-immunoprecipitate in MT tumors. A, MT tumors (1 mg) were immunoprecipitated with anti-Src or anti-Muc1 and immunoblotted for c-Src (*upper panel*) or immunoblotted for Muc1 (*ower panel*). MT tumor lysate (100 μg) was used as positive control for c-Src and Muc1 expression. **B,** MT and MMT tumors (1 mg) were immunoprecipitated with agarose-conjugated CT2 and immunblotted for eSrc (*upper* panel) and for Muc1 (*lower panel*).
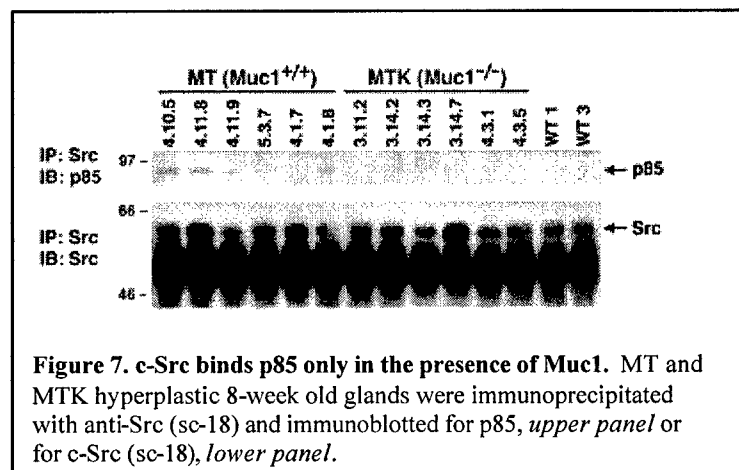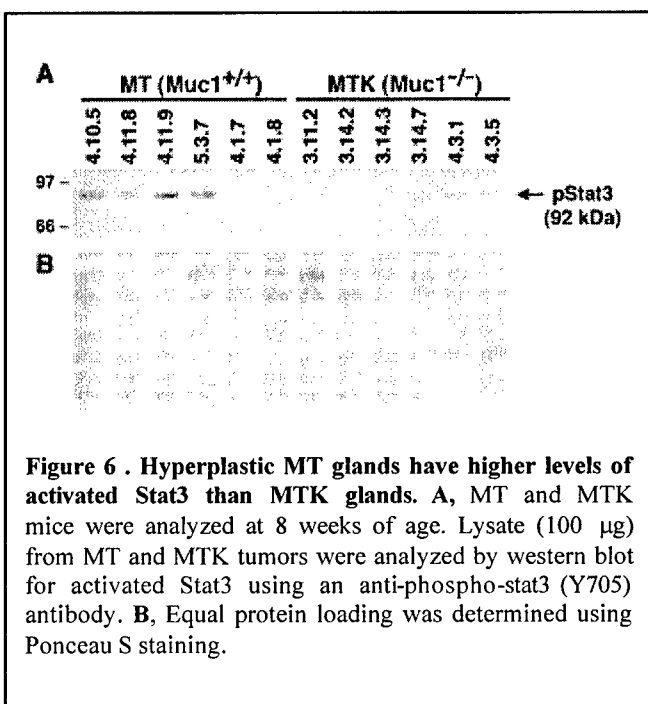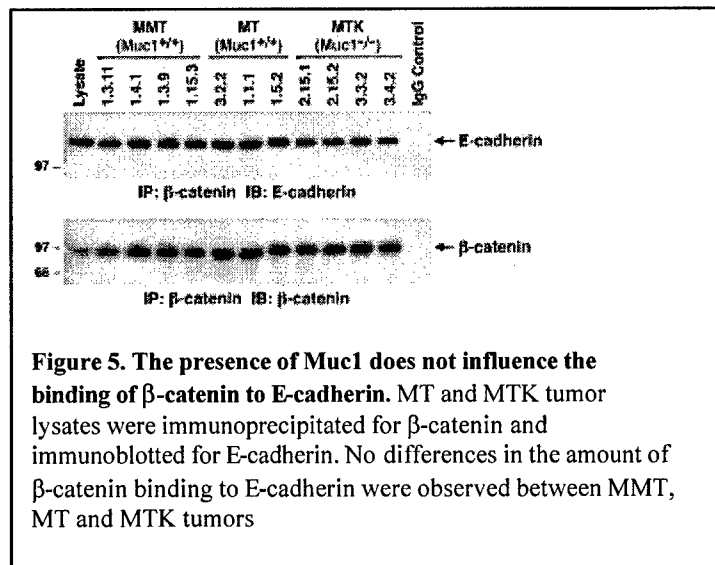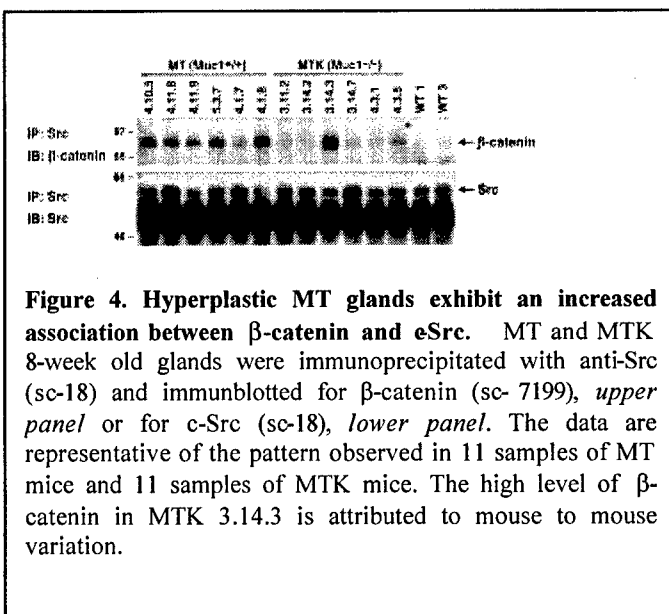

Figure 2. Muc1 and c-Src colocalize in MT tumors. MT tumors were stained for Muc1 (CT2) on green and c-Src (2-17) in red. Sections were examined at 250X magnification using a Zeiss 510 laser scanning microscope. Colocalization is depicted in yellow in overlay panel.

Figure 3. 8 week old MT and MTK mice exhibit similar levels of c-Src kinase activity. c-Src was immunoprecipitated from lysates of MT and MTK samples at the initial stages of tumorigenesis when mice are at 8 weeks of age. **A,** kinase activity was measured by the amount of γ ^{32}P ATP incorporated in a c-Src substrate using a kit from Upstate Biotechnology (Lake Placid, NY). **B,** as a control for the amount of c-Src, duplicate immunoprecipitations were carried out and assayed by immunoblotting for c-Src. There was no significant difference in the mean of c-Src activity between MT and MTK animals.

Figure 4. Hyperplastic MT glands exhibit an increased association between β-catenin and eSrc. MT and MTK 8-week old glands were immunoprecipitated with anti-Src (sc-18) and immunblotted for β-catenin (sc- 7199), *upper panel* or for c-Src (sc-18), *lower panel*. The data are representative of the pattern observed in 11 samples of MT mice and 11 samples of MTK mice. The high level of β-catenin in MTK 3.14.3 is attributed to mouse to mouse variation.

Figure 5. The presence of Muc1 does not influence the binding of β-catenin to E-cadherin. MT and MTK tumor lysates were immunoprecipitated for β-catenin and immunoblotted for E-cadherin. No differences in the amount of β-catenin binding to E-cadherin were observed between MMT, MT and MTK tumors

Figure 6 . Hyperplastic MT glands have higher levels of activated Stat3 than MTK glands. A, MT and MTK mice were analyzed at 8 weeks of age. Lysate (100 μg) from MT and MTK tumors were analyzed by western blot for activated Stat3 using an anti-phospho-stat3 (Y705) antibody. **B,** Equal protein loading was determined using Ponceau S staining.

Figure 7. c-Src binds p85 only in the presence of Muc1. MT and MTK hyperplastic 8-week old glands were immunoprecipitated with anti-Src (sc-18) and immunoblotted for p85, *upper panel* or for c-Src (sc-18), *lower panel.*

A

IP: FAK IB: CT2

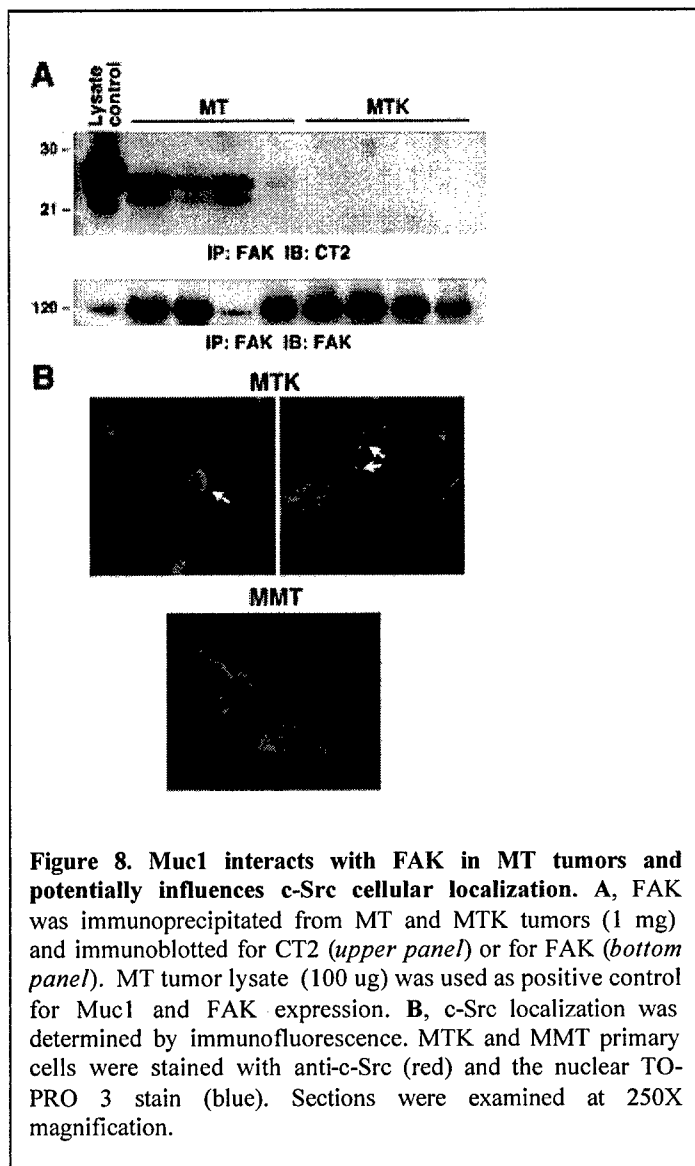IP: FAK IB: FAK

B

MTK

MMT

Figure 8. Muc1 interacts with FAK in MT tumors and potentially influences c-Src cellular localization. A, FAK was immunoprecipitated from MT and MTK tumors (1 mg) and immunoblotted for CT2 (*upper panel*) or for FAK (*bottom panel*). MT tumor lysate (100 ug) was used as positive control for Muc1 and FAK expression. **B,** c-Src localization was determined by immunofluorescence. MTK and MMT primary cells were stained with anti-c-Src (red) and the nuclear TO-PRO 3 stain (blue). Sections were examined at 250X magnification.
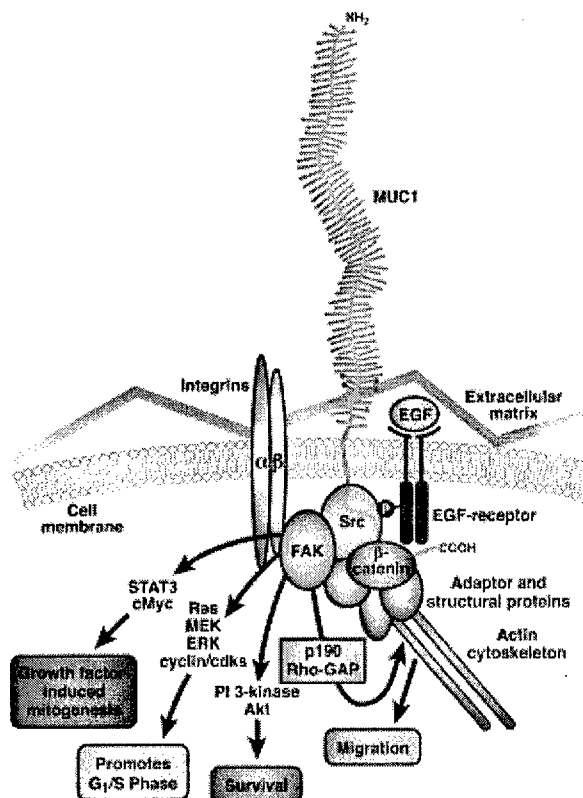
Figure 9. Model illustrating how Muc1 could be influencing c-Src signaling. We hypothesize that the overexpression of Muc1 by tumor cells may potentiate c-Src signaling by bringing it closer to its downstream targets such as β-catenin, FAK, and p85 at the cell membrane. Activation of c-Src influences various aspects of cell behavior including growth, proliferation, survival and migration.

KEY RESEARCH ACCOMPLISHMENTS

- c-Src levels and activity were higher in MT and MMT tumors than in normal mammary glands.
- Muc1 co-localizes and physically interacts with c-Src in MT and MMT tumors, corroborating previous *in vitro* observations and thus substantiating a physiological relevance for the association.
- We did not observe a significant effect of Muc1 expression on c-Src activity as determined by kinase assays comparing MT and MTK samples. However, Muc1 expression was found to influence c-Src signaling and localization.
- Muc1 expression promotes the association between c-Src and β-catenin in MT hyperplastic glands.
- Muc1 expression does not decrease the association of β-catenin and E-cadherin.
- Hyperplastic MT glands expressing Muc1 have higher levels of activated Stat3 than MTK glands.
- c-Src associates with the p85 subunit of PI3K only in the presence of Muc1.
- Muc1 directly associates with FAK and can influence c-Src cellular localization.
- MUC1 overexpression in MMTV-MUC1 transgenic mice results in the stochastic formation of mammary gland tumors.
- MUC1 overexpression results in sustained glandular differentiation in multiparous MMTV-MUC1 animals.
- MUC1 and β-catenin interact in MMTV-MUC1 tumors and hyperplasis and in MMTV-MUC1 lactating glands.
- Increased pErk1 and pErk2 activation in the MMTV-MUC1 mammary glands.
- MMTV-MUC1 transgenics exhibit delayed post-lactational involution correlating with the presence of large secretory ducts as determined by whole mount and histological analysis.
- MMTV-MUC1 transgenic glands exhibit elevated WAP expression at day 6 of post-lactational involution.
- Involuting MMTV-MUC1 glands display decreased levels of apoptosis compared with control wildtype glands.
- MMTV-MUC1 glands exhibit increased levels of pErk2 activation at day 8 of post-lactational involution.
- Involuting MMTV-MUC1 glands display increased levels of Erk1/2 activation at day 2 of involution and persistent Erk2 activation at day 8 of involution.

REPORTABLE OUTCOMES

DEGREES

- I, Azzah Al Masri, obtained the degree of doctor of philosophy in Biomedical Sciences/ Molecular Biology from the Mayo Clinic College of Medicine (June 2004).

PUBLICATIONS

- Azzah Al Masri, Joyce A. Schroeder, Melissa C. Adriance, Melissa C. Thompson, and Sandra J. Gendler. *MUC1 overexpression induced mammary gland tumors and delayed postlactational involution.* Oncogene (2004) Vol. 23 pp.5739–5747.

- Azzah Al Masri and Sandra J. Gendler. *Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis.* Oncogene (2005) May 9; [Epub ahead of print].

- Azzah Al Masri. *The Role of MUC1 Signaling in Breast Cancer.* A thesis submitted to the faculty of the Mayo Graduate School. April 2004.

POSTERS AND ORAL PRESENTATIONS

- Azzah Al Masri, Joyce A. Schroeder, Melissa C. Adriance, Melissa C. Thompson, and Sandra J. Gendler. *MUC1 overexpression induced mammary gland tumors and delayed postlactational involution.* Abstract and poster at the AACR Mouse Models Meeting. Lake Buena Vista, FL. 2/19/2003-2/22/03. **I gave an oral presentation of the data at the AACR meeting.**

- Azzah Al Masri and Sandra J. Gendler. *Understanding the role of Muc1 in PyV MT induced tumorigenesis.* Abstract and poster at the FASEB meeting: Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis and Tumorigenesis. Tucson, AZ. 8/2/2003-8/7/2003. **I gave an oral presentation of the data at the FASEB meeting.**

- Azzah Al Masri. *The Role of MUC1 Signaling in Breast Cancer.* Thesis defense oral presentation. Mayo Clinic Scottsdale, AZ. 5/22/2004.

CELL LINES AND MOUSE MODELS

- Generation of PyV MT transgenics lacking Muc1 on the FVB background in addition to bitransgenics expressing the mutant form of MUC1ΔCT and PyV MT on a Muc1 knockout backgound.

- Generation of MT, MMT and MTK cell lines derived from transgenic mouse mammary glands.

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CONCLUSIONS

The MMTV-PyV MT mouse model was used to develop a better understanding of the signaling role of Muc1 in tumorigenesis. The MMTV-PyV MT model has proven invaluable in dissecting the signal transduction pathways that are altered during mammary gland transformation. In the MMTV-PyV MT tumors, Muc1 expression is dramatically increased [38]. Additionally, the significant delay in tumor progression in the MMTV-PyV MT x Muc1$^{-/-}$ defined a functional role for Muc1 in PyV MT-induced tumorigenesis [6]. We analyzed MMTV-PyV MT mammary tumors on a wildtype background compared with tumors of MMTV-PyV MT mice on a Muc1 null background to specifically delineate the effect of Muc1 on PyV MT-induced mammary tumorigenesis. We identified an *in vivo* association between Muc1 and c-Src in PyV MT-induced tumors. We proposed two mechanisms by which Muc1 could promote tumorigenesis. Our findings rule out the first mechanism by which Muc1 may directly influence c-Src kinase activity and promote c-Src activation by inducing conformational changes or recruiting c-Src activating molecules. Instead our data indicate that Muc1 contributes to PyV MT signaling by influencing c-Src signaling in promoting its association with downstream signaling molecules. Muc1 promotes the association of c-Src with signaling targets such as β-catenin and the p85 subunit PI3K. Additionally, an increase in the levels of activated Stat3 and in some animals activated Erk2 in the PyV MT tumors was observed in the presence of Muc1. We demonstrate a novel association between Muc1 and a known Src substrate FAK, which potentially promotes the peripheral targeting of activating c-Src. In primary cell cultures from mammary epithelial cells derived from MT and MTK mice, we observed that in the absence of Muc1, Src is commonly found at the perinuclear region of the cell in contrast to the cytoplasmic and peripheral localization of Src in the presence of Muc1. These findings provide insights into the possible mechanism for the significant delay in tumor progression that is observed in the absence of Muc1. We suggest that the interaction of Muc1 with c-Src, a key player in PyV MT transformation, promotes the binding of c-Src to its downstream targets and influences its cellular localization. Our understanding of the critical role of Muc1 signaling in PyV MT-induced oncogenesis will be invaluable in delineating the effect of MUC1 overexpression in human breast cancer.

The question of whether MUC1 overexpression is a cause or a consequence is at the heart of understanding the role of MUC1 in cancer. We now show that the overexpression of MUC1 in the MMTV-MUC1 mouse model is directly involved in the *in vivo* transformation of the mammary gland. We have found that prolonged expression of MUC1 in the mammary gland results in the stochastic development of unifocal tumors. Coincident with the development of frank neoplasia, prolonged expression of MUC1 inhibited post-lactational involution by the inhibition of apoptosis. As such, increased MUC1 expression is not merely a byproduct of transformation, but can itself promote transformation in physiological context. We demonstrate a decrease in apoptosis in the involuting MMTV-MUC1 transgenic glands in comparison to the wildtype glands. We also observed elevated Erk activation in correlation with MUC1 overexpression.

MUC1 has long been recognized as a tumor-associated antigen. More than 90% of human breast cancers overexpress MUC1. Our understanding of the biology of MUC1 and its contribution to malignant transformation is constantly evolving. The complexity of the structure of MUC1 mirrors the complexity in the function of this molecule. Burgeoning evidence indicates that

MUC1 overexpression is not merely a byproduct of transformation. It contributes to the survival of tumor cells, promoting proliferation and invasion. The fact that overexpression of MUC1 can

itself promote transformation in a physiological context sheds new light on the biology of this molecule. MUC1 is considered a therapeutic target in immunotherapy studies and MUC1-based therapy is currently at different stages of clinical testing. However, the interactions of this molecule with key signaling proteins serve as targets for future drug development that potentially influence the differentiation, proliferation and migration of tumor cells.

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Azzah Al Masri, Joyce A. Schroeder, Melissa C. Adriance, Melissa C. Thompson, and Sandra J. Gendler. *MUC1 overexpression induced mammary gland tumors and delayed postlactational involution.* Oncogene (2004) Vol. 23 pp.5739–5747.

Azzah Al Masri and Sandra J. Gendler. *Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis.* Oncogene (2005) May 9; [Epub ahead of print].

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ABSTRACTS

- **Submitted and presented at AACR Mouse Models Meeting. Lake Buena Vista, FL. 2/19/2003-2/22/03**

Title: MUC1 overexpression induced mammary gland tumors and delayed involution.

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Abstract

MUC1 is a transmembrane mucin that was initially cloned from malignant mammary epithelial cells as a tumor antigen. More than 90% of human breast carcinomas and metastases overexpress aberrantly glycosylated MUC1. Recent studies have shown that the MUC1 cytoplasmic domain interacts with β-catenin, erbB receptors, src, GSK3β, p120[ctn], Grb2 and Protein Kinase Cδ, possibly potentiating cell proliferation and disassembly of cell-cell adhesion. Transgenic and knockout mice have provided insight into the role of MUC1 in tumor development. Transgenic mice expressing the polyomavirus middle T antigen under control of the MMTV promoter exhibited a significant delay in tumor progression and a trend towards decreased metastasis when bred onto a Muc1 knockout background. Lack of Muc1 expression also caused a significant

delay in tumor onset in the MMTV-Wnt-1 model of breast cancer. These studies led to the hypothesis that MUC1 plays an integral role in tumor progression. The objective of this study is

to gain a better understanding of the physiological role of MUC1 in breast cancer. In order to do that, we generated transgenic mice that overexpress either full-length or cytoplasmic domain-deleted human MUC1 using the MMTV promoter to direct the expression of the transgene to the mammary gland. Long-term analysis of MUC1 transgenic mice led to the finding that overexpression of full-length MUC1 but not the cytoplasmic domain-deleted MUC1 resulted in stochastic formation of mammary gland tumors that metastasized to the lungs in multiparous females. Tumor formation was accompanied by a failure of multiparous glands to dedifferentiate and involute. Additionally, we have determined that this lack of involution can be observed in non-tumor bearing mammary glands as well, in that post-lactational regression is suppressed by MUC1 overexpression. We found that the delay in mammary involution in the MUC1 transgenics correlated with elevated WAP expression and pErk2 activation. Our findings indicate that MUC1 functions as a weak oncogene whereby the cytoplasmic tail of MUC1 acts as a scaffolding protein to coordinate and enhance multiple growth-promoting and oncogenic signals.

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- **Submitted and presented at the FASEB meeting: Growth Factor Receptor Tyrosine Kinases in Mitogenesis, Morphogenesis and Tumorigenesis. Tucson, AZ. 8/2/2003-8/7/2003**

Title: The Role of Muc1 in PyV MT Tumorigenesis

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Background: MUC1 is an integral membrane mucin glycoprotein that is normally expressed on the apical surface of most simple, secretory epithelia as well as a number of hematopoietic cells. Overexpression of aberrantly glycosylated MUC1 is a hallmark of many carcinomas including 90% of breast carcinomas. Notably, a number of observations point to a role for MUC1 in signal transduction. Recently, MUC1 has been shown to interact with the c-Src tyrosine kinase *in vitro*, whereby c-Src phosphorylates the MUC1 cytoplasmic domain at a YEKV motif. c-Src is an extensively studied nonreceptor tyrosine kinase implicated in mammary tumorigenesis. Also, aberrant activation of c-Src tyrosine kinase has been characterized in a large proportion of human breast carcinomas. In our lab, the role of mouse Muc1 in tumorigenesis and disease progression was assessed in the polyomavirus middle T antigen (PyV MT)-induced spontaneous mammary tumor model (MMTV-mTag). mTag transgenic mice crossed onto a Muc1 null background exhibited a significant delay in mammary tumor progression. Interestingly, c-Src has been shown to interact with PyV MT, and to play an integral and indispensable role in the development of metastatic mammary tumors in the mTag transgenic mice. It may be that the lack of Muc1 affects c-Src activity negatively, resulting in slower tumor progression.

Hypothesis: **MUC1 mediates molecular interactions between oncogenic kinases and other signaling proteins that contribute significantly to transformation.**

Aims: To identify specific proteins that associate with MUC1 and induce signaling that potentiates tumor development.

Results:
- The interaction between MUC1 and Src has been confirmed in the mammary tumors of the mTag transgenic mice by reciprocal co-immunoprecipitation experiments.
- Immunofluorescence analysis indicates colocalization between MUC1 and c-Src in the mTag tumors, thereby verifying the interaction between MUC1 and c-Src.
- Preliminary findings using *in vitro* kinase assays suggest decreased Src kinase activity in mTag tumors lacking Muc1.
- An interaction between MUC1 and the p85 subunit of PI3K has been identified by co-immunoprecipitation in the breast cancer line T47D and studies are underway to characterize such an interaction in the mTag tumors.

Conclusions:
It is likely that MUC1 functions to recruit c-Src and other signaling molecules that are essential for polyoma middle T-induced transformation. Further studies will determine how the interactions between MUC1 and the molecules involved in PyV MT signaling contribute to tumor progression.

PERSONNEL

Azzah Al Masri. 100% effort and salary. Azzah was responsible for 100% of the experiments described in this report.

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ORIGINAL PAPER

Muc1 affects c-Src signaling in PyV MT-induced mammary tumorigenesis

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MUC1 is an integral membrane mucin glycoprotein that is normally expressed on the apical surface of most simple, secretory epithelia and hematopoietic cells. Overexpression of aberrantly glycosylated MUC1 is a hallmark of many carcinomas including 90% of breast carcinomas. MUC1 has been shown to bind to c-Src tyrosine kinase in vitro, whereby c-Src phosphorylates the MUC1 cytoplasmic domain at a YEKV motif. c-Src is an extensively studied nonreceptor tyrosine kinase implicated in mammary tumorigenesis. Previously, mouse mammary tumor virus-driven polyoma middle T-antigen (MMTV-PyV MT) transgenic mice crossed onto a Muc1 null background exhibited a significant delay in tumor progression. c-Src has been shown to interact with PyV MT, and to play an integral and indispensable role in MMTV-PyV MT-induced mammary tumorigenesis. Here, we determine the effect of Muc1 expression on c-Src activation and signaling. Examination of MMTV-PyV MT glands on a wild-type or Muc1 null background demonstrates that Muc1 expression promotes c-Src signaling by influencing its association with known substrates such as the p85 subunit of phosphatidylinositol 3-kinase and β-catenin. These findings may provide a mechanism for the delay in tumor progression that is observed in the absence of Muc1.
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Keywords: mucin; oncogene; transgenic mice; breast cancer; mammary gland

Introduction

MUC1 (MUC1 in humans, Muc1 in mice) is the founding member of a large and evolutionarily conserved family of proteins known as mucins (Gendler and Spicer, 1995; Gendler, 2001; Hollingsworth and Swanson, 2004). Mucins are broadly defined as proteins with extensive O-linked glycosylation, which constitutes 50%

of the total molecular mass. MUC1 is a transmembrane mucin with a large, heavily glycosylated extracellular domain and a highly conserved cytoplasmic tail. It is normally expressed on the apical surface of most simple secretory epithelia and several hematopoietic cell lineages (Gendler, 2001).

MUC1 was initially cloned from mammary and pancreatic carcinomas as a tumor antigen (Gendler et al., 1987, 1990; Lan et al., 1990; Ligtenberg et al., 1990; Wreschner et al., 1990). More than 90% of human breast cancers overexpress aberrantly glycosylated MUC1 (Zotter et al., 1988). In cancer cells, the pattern of MUC1 expression is altered such that it is no longer restricted to the apical surfaces; instead, it is expressed intracellularly and around the entire cell surface (Hilkens et al., 1995). Accumulating evidence suggests a functional role for MUC1 in transformation. It has been suggested that MUC1 contributes to tumor progression by functioning as an antiadhesive molecule, thereby destabilizing cell–cell and cell–matrix connections (Wesseling et al., 1995, 1996). Other findings suggest that MUC1 acts as a proadhesive molecule, facilitating the metastatic process by allowing migrating cells to reattach (Regimbald et al., 1996; Kam et al., 1998). Additionally, numerous observations point to a role for MUC1 in signal transduction. The 72-amino-acid tail of MUC1 contains seven tyrosines, six of which are 100% conserved across mammalian species (Spicer et al., 1995a). The cytoplasmic tail of MUC1 is phosphorylated on tyrosine residues in epithelial cell lines and mouse mammary glands; it contains potential docking sites for SH2-containing proteins and several kinase recognition sites (Zrihan-Licht et al., 1994; Mockensturm-Gardner and Gendler, 1998; Schroeder et al., 2001). Indeed, MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as EGFR, erbB2, erbB3, erbB4, c-Src, PKCδ, Grb2, β-catenin, GSK3β, and p120^{ctn} (Pandey et al., 1995; Yamamoto et al., 1997; Li et al., 1998, 2001a, b; Li and Kufe, 2001; Schroeder et al., 2001; Ren et al., 2002). Yet in spite of MUC1 being recognized as a tumor-associated antigen, its precise function in tumorigenesis and disease progression remains elusive.

As mentioned above, c-Src is among the oncogenic proteins that interact with the cytoplasmic tail of MUC1 (Li et al., 2001a, b). c-Src is a well-characterized nonreceptor tyrosine kinase implicated in mammary tumorigenesis. c-Src is located within adhesion plaques and at cadherin-mediated cell–cell junctions in epithelial

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cells (Jones *et al.*, 2000). Aberrant activation of c-Src tyrosine kinase is a characteristic of a large proportion of human breast carcinomas (Ottenhoff-Kalff *et al.*, 1992). High levels of c-Src result in the activation of multiple mitogenic signaling pathways including the Ras/MAPK pathway (Penuel and Martin, 1999). Figure 9 depicts the cellular effects downstream of c-Src activation promoting growth, mitogenesis, survival, and migration. Previous *in vitro* studies have shown that MUC1 interacts with the c-Src tyrosine kinase, whereby c-Src phosphorylates the MUC1 cytoplasmic domain at a YEKV motif (Li *et al.*, 2001a).

It is believed that MUC1 competes with E-cadherin for β-catenin binding and therefore is involved in disrupting cell adhesion complexes (Ligtenberg *et al.*, 1992; Wesseling *et al.*, 1995). Previously, it has been shown that, *in vitro*, β-catenin interacts with MUC1 at an SXXXXXSSL site present in the cytoplasmic tail of MUC1 (Yamamoto *et al.*, 1997). GSK3β also has been shown to bind directly to MUC1 at a DRSPY site adjacent to that for β-catenin, hence decreasing the interaction of MUC1 with β-catenin (Li *et al.*, 1998). On the other hand, the interaction between MUC1 and c-Src results in the phosphorylation of MUC1 at the YEKV motif located between sites involved in interactions with GSK3β and β-catenin. Phosphorylation of MUC1 provides a binding site for c-Src SH2 domain, and in turn inhibits the interaction between MUC1 and GSK3β (Li *et al.*, 2001a). Therefore, inhibition of the association of MUC1 with GSK3β increases the binding of MUC1 and β-catenin.

A better understanding of the function of MUC1 in tumorigenesis has been derived from transgenic and knockout mouse models. Lack of Muc1 expression leads to a significant delay in tumor onset in the mouse mammary tumor virus (MMTV)-Wnt-1 mouse model of breast cancer, suggesting that Muc1 is involved in the initiation stages of cancer in that β-catenin-dependent model (Schroeder *et al.*, 2003). We have recently shown that MUC1 overexpression promotes *in vivo* transformation of the mammary gland, establishing that MUC1 is not merely a by-product of transformation but can itself promote transformation in a physiological context (Schroeder *et al.*, 2004). To elucidate the role of MUC1 in tumor progression and metastasis, we crossed MMTV-driven polyoma middle T-antigen (MMTV-PyV MT) transgenic mice onto a Muc1 null background (Spicer *et al.*, 1995b). While MMTV-PyV MT transgenic mice developed multifocal mammary tumors with a high frequency of lung metastasis (Guy *et al.*, 1992), tumor progression was significantly delayed in Muc1 null/ MMTV-PyV MT transgenics (Spicer *et al.*, 1995b). The high transforming activity of the PyV MT antigen is dependent on the interaction of the PyV MT with the Src family of kinases (c-Src, c-Yes, and Fyn), and it has been shown that activation of c-Src is essential for the induction of mammary tumors in the MMTV-PyV MT transgenic mice (Guy *et al.*, 1994). The delayed mammary tumor progression in the MMTV-PyV MT transgenic mice lacking Muc1 and the functional interaction between MUC1 and c-Src suggest a signaling

role for Muc1 in MMTV-PyV MT tumorigenesis. We hypothesize that MUC1 is an important contributor to transformation and functions as a target for oncogenic kinases and signaling proteins.

The MMTV-PyV MT mouse model is used to develop a better understanding of the signaling role of Muc1 in tumorigenesis. The MMTV-PyV MT model has proven invaluable in dissecting the signal transduction pathways that are altered during mammary gland transformation. PyV MT antigen lacks intrinsic biochemical activity, yet it exerts its oncogenic potential by hijacking the normal regulatory pathways that govern cellular proliferation and survival. PyV MT modulates the activities of a variety of key regulatory proteins such as phosphatase 2A, tyrosine kinases of the Src family, phosphatidylinositol 3-kinase (PI3K), Shc, and phospholipase C-γ1 (PLC-γ1) (Dilworth, 1995).

In this study, we examined the effect of Muc1 expression on c-Src signaling in the MMTV-PyV MT model in an effort to discern the mechanism for the delay in tumor progression that is observed in the absence of Muc1. We identified an *in vivo* association between Muc1 and c-Src in the PyV MT-induced mammary tumors. We show that Muc1 expression promotes the interaction of c-Src with key signaling proteins and affects downstream Src signaling as suggested in the model shown in Figure 9. The data suggest that Muc1 functions to promote PyV MT signaling mediated by c-Src activation.

Results

c-Src levels are increased in the MT and MMT tumors

We have previously shown that MMTV-PyV MT transgenic mice exhibit a significant delay in tumor progression and a trend toward decreased metastasis when bred onto a Muc1 knockout background. To understand the mechanism by which Muc1 affects PyV MT-induced tumorigenesis, we examined the effect of Muc1 expression on c-Src. The intracellular tyrosine kinase, c-Src, plays a pivotal role in mediating the oncogenic effects of the PyV MT antigen. As described in Table 1, the mouse models used in this study include MMTV-PyV MT transgenic mice either on a wild-type background (MT) or a Muc1 knockout background (MTK). We also crossed the MMTV-PyV MT mice with mice expressing the human MUC1 transgene on a mouse Muc1 wild-type background to generate MMT bitransgenics. Normal mammary glands from age-matched wild-type mice were used as controls for PyV

Table 1 List and genotype of mouse models used

Mouse models	Genotype
MT	MMTV-PyV MT/Muc1$^{+/+}$
MTK	MMTV-PyV MT/Muc1$^{-/-}$
MMT	MMTV-PyV MT/Muc1$^{+/+}$/MUC1$^{+/0}$

MT-induced tumors. Figure 1 shows a marked increase in the level of c-Src expression in the tumors from MT and MMT mice compared with normal mammary glands. Interestingly, the expression of other c-Src like kinases was evident only in the tumor samples.

Muc1 co-immunoprecipitates with c-Src in PyV MT-induced tumors

We characterized the association between Muc1 and c-Src in the PyV MT-induced mammary tumors. Mammary gland tumor lysates from MT mice were immunoprecipitated with antibodies to c-Src or to the cytoplasmic tail of Muc1. The immunoprecipitates were then analysed by immunoblotting for c-Src (Figure 2a, upper panel) and Muc1 (Figure 2a, lower panel). Reciprocal co-immunoprecipitations confirmed an interaction between c-Src and Muc1 in the mammary tumors from MT mice (Figure 2a). On a Western blot, the cytoplasmic tail of Muc1 separates from the rest of the protein and runs within the 25–30 kDa range. The variability in the molecular mass of Muc1 is attributed to the differential glycosylation of the protein.

The molecular mass of c-Src is 60 kDa and that of immunoglobulin heavy chain is around 55 kDa. In order to eliminate the possibility of contamination with immunoglobulin heavy chain, we carried out the Muc1 immunoprecipitations using an immobilized Muc1 antibody. Immunoblotting for c-Src verified that c-Src forms a complex with Muc1 in tumor lysates from MT and MMT mice (Figure 2b).

Muc1 and c-Src colocalize in the MT tumors

To further verify the interaction between c-Src and Muc1 and to determine the location of this complex, we used confocal microscopy to analyse Muc1/c-Src localization. Mammary tumors from MT mice were stained with antibodies to Muc1 (green) and c-Src (red). Muc1 was localized to the apical membrane while c-Src was localized throughout the cell membrane and throughout the cytoplasm. An overlay of their staining patterns revealed that they are colocalized mainly at the apical membrane (Figure 3).

Determining the effect of Muc1 on c-Src activity

We next investigated the effect of the Muc1/c-Src interaction on PyV MT-mediated transformation. We proposed two mechanisms by which Muc1 could promote tumorigenesis. One is that Muc1 may directly influence c-Src kinase activity and promote c-Src activation by inducing conformational changes or recruiting c-Src activating molecules. Alternatively, Muc1 may influence c-Src signaling by promoting its association with downstream signaling molecules.

We investigated the first mechanism where Muc1 could influence PyV MT-induced tumorigenesis by regulating c-Src activity. In order to address the question of whether Muc1 modulates c-Src kinase activity, we compared c-Src activity in the hyperplastic glands from MT mice ($n = 6$) with hyperplastic glands from MTK mice ($n = 6$), which lack Muc1. The activity of c-Src immunoprecipitated from mouse mammary glands was measured by [^{32}P]ATP incorporation into a peptide substrate. As a control for the amount of c-Src, duplicate immunoprecipitations were carried out and assayed by immunoblotting for c-Src. Densitometry of the c-Src blots was used to normalize the kinase activity to the amount of immunoprecipitated c-Src. We compared c-Src kinase activity in (MT and MTK samples) at the initial stages of tumorigenesis when mice were 8 weeks of age. It may be that the presence of Muc1 plays a role at the initial stages of tumorigenesis, hence we might see a greater difference in c-Src kinase
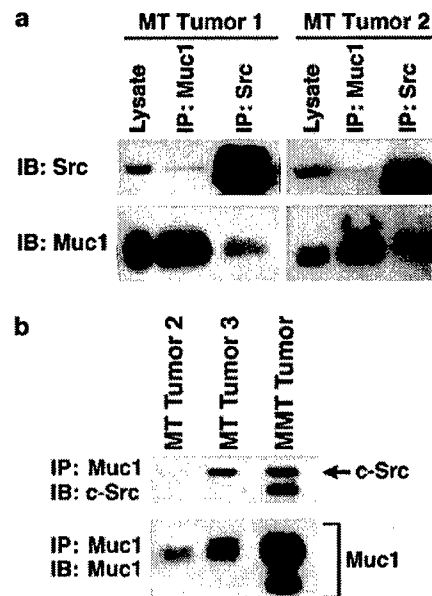

Figure 2 Muc1 and c-Src co-immunoprecipitate in tumors from MT mice. (a) Tumors (1 mg) from MT mice were immunoprecipitated with anti-Src or anti-Muc1 (CT2) and immunoblotted for c-Src (upper panel) or immunoblotted for Muc1 (lower panel). Tumor lysate (100 μg) from MT mice was used as positive control for c-Src and Muc1 expression. (b) Tumors (1 mg) from MT and MMT mice were immunoprecipitated with agarose-conjugated CT2 and immunoblotted for c-Src (upper panel) and for Muc1 (lower panel)
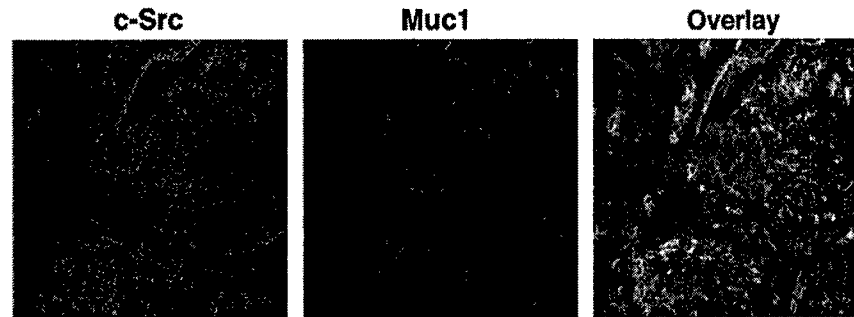

Figure 1 Increased c-Src levels are observed in tumors from MMT and MT mice. Lysates (100 μg) of tumors from MMT and MT mice and mammary glands of wild-type females were analysed by Western blot for c-Src (sc-18) expression levels. The expression of other c-Src-like kinases is detected in the tumor lysates

4

Figure 3 Muc1 and c-Src colocalize in tumors from MT mice. Tumors from MT mice were stained for Muc1 (CT2) in green and c-Src (2–17) in red. Sections were examined at × 250 magnification using a Zeiss 510 laser scanning microscope. Colocalization is depicted in yellow in the overlay panel

activity between MT and MTK mice at an earlier time point. We did not observe a significant difference in c-Src kinase activity between the hyperplastic glands of MT and MTK mice (data not shown). These observations suggest that the delayed tumorigenesis was not a consequence the inability of Muc1 to functionally activate c-Src tyrosine kinase. As such, we ruled out the first suggested mechanism for the role of Muc1 in PyV MT signaling, that of direct activation.

Muc1 expression promotes the association between c-Src and β-catenin

We determined the effect of Muc1 on several signaling pathways downstream of c-Src. We directed our efforts toward analysis of c-Src downstream signaling pathways in the presence or absence of Muc1. Among the c-Src substrates that we investigated is β-catenin. β-catenin is a component of the E-cadherin-mediated cell–cell junctions. The tyrosine phosphorylation of β-catenin by c-Src has been shown to inhibit the binding of β-catenin to E-cadherin, disrupt cell–cell adhesions, and promote cancer cell invasion. We investigated the association between c-Src and β-catenin in the hyperplastic glands from MT and MTK mice by immunoprecipitation for c-Src. Blotting for β-catenin revealed an increased association between c-Src and β-catenin in the presence of Muc1 (Figure 4). Only one MTK gland (3.14.3) showed a c-Src/β-catenin association that was comparable to Muc1-containing MT glands.

Muc1 expression does not decrease the association of β-catenin and E-cadherin

Since Muc1 affects β-catenin interactions in PyV MT-induced tumors, we were interested in determining whether Muc1 expression would influence the association between β-catenin and E-cadherin. To address this question, we immunoprecipitated β-catenin from tumors from MT, MMT, and MTK mice and immunoblotted for E-cadherin to assay for β-catenin/E-cadherin association. According to the model proposed by Kufe and co-workers (Li *et al.*, 1998), we expected to see decreasing β-catenin/E-cadherin association in the
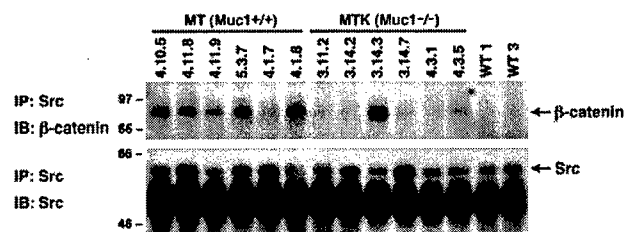

Figure 4 Hyperplastic glands from MT mice exhibit an increased association between β-catenin and c-Src. Glands from 8-week-old MT and MTK mice were immunoprecipitated with anti-Src (sc-18) and immunoblotted for β-catenin (sc-7199) (upper panel) or for c-Src (sc-18) (lower panel). The data are representative of the pattern observed in 11 samples of MT mice and 11 samples of MTK mice. The high level of β-catenin in MTK 3.14.3 is attributed to mouse to mouse variation
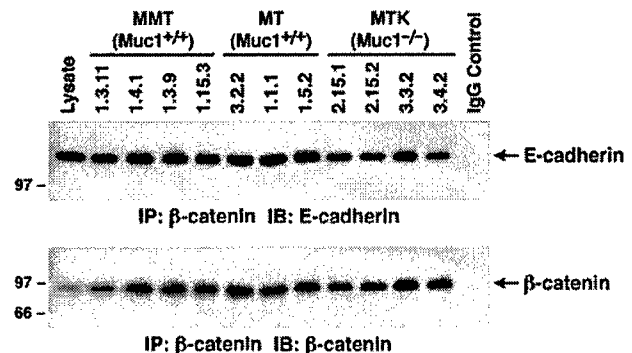

Figure 5 The presence of Muc1 does not influence the binding of β-catenin to E-cadherin. Tumor lysates from MT and MTK mice were immunoprecipitated for β-catenin and immunoblotted for E-cadherin. No differences in the amount of β-catenin binding to E-cadherin were observed in tumors from MMT, MT, and MTK mice

tumors from MMT and MT mice compared to tumors from MTK mice. However, we saw no difference in the level of β-catenin/E-cadherin association between tumors from MT and MTK mice (Figure 5). This likely reflects differences between the interactions influencing

cell adhesion *in vitro* and those observed in the physiological context of the mouse mammary tumors.

Hyperplastic MT glands expressing Muc1 have higher levels of activated Stat3 than MTK glands

Stat3 activation is a known downstream event of c-Src signaling. Stat3 is activated in a wide variety of human tumors, where it is known to enhance tumor cell proliferation and prevent apoptosis (Calo *et al.*, 2003). MT and MTK mice were analysed at 8 weeks of age when the mammary glands are hyperplastic but not frankly tumorigenic. Using an antibody specific to the activated form of Stat3 (anti-phospho-Stat3 Y705), we observed higher levels of activated pStat3 in the MT mice compared with the MTK mice (Figure 6). Muc1 expression may be enhancing the activation of Stat3, possibly by its association with EGFR and c-Src, which have been shown to increase Stat3 activation.

c-Src associates with the p85 subunit of PI3K only in the presence of Muc1

The p85 subunit of PI3K is among the key signaling molecules that associate with the PyV MT antigen (Talmage *et al.*, 1989; Freund *et al.*, 1992). Activation of PI3K signaling influences cell proliferation, growth, survival, and cytoskeletal organization (Whitman *et al.*, 1985; Courtneidge and Heber, 1987; Cantley, 2002). Binding of p85 to the PyV MT antigen is a necessary event for PyV MT-induced tumorigenesis and may stimulate cell survival (Webster *et al.*, 1998). The association between c-Src and the p85 subunit of PI3K is thought to modulate the localization of activated c-Src at the cell membrane (Fincham *et al.*, 2000). We investigated whether the presence of Muc1 influenced the interaction of Src with p85. The interaction between c-Src and the p85 subunit of PI3K kinase was evident only in the hyperplastic glands from 8-week-old MT mice and not in the MTK mice (Figure 7). However, we did not observe changes in pAkt, a downstream target of

p85 signaling between MT and MTK samples (data not shown). The presence of Muc1 facilitates the interaction between c-Src and p85, and as such may promote tumor progression in the MT transgenic mice.

Muc1 directly associates with FAK and can influence c-Src localization

The intracellular localization of c-Src is critical for mediating its downstream signaling effects. In light of the fact that the presence of Muc1 was associated with enhancing c-Src interaction with its downstream targets, we next investigated the effect of Muc1 on c-Src localization. One substrate of c-Src is the focal adhesion kinase (FAK), which is localized to focal adhesions at the cell surface. Immunoprecipitation for FAK in tumor samples from MT and MTK mice revealed that Muc1 was in a complex with FAK (Figure 8a). Association of Muc1 with FAK and c-Src could promote peripheral localization of c-Src, which could then more readily activate FAK.

To better understand the effect of Muc1 expression on c-Src localization, we generated primary epithelial cell cultures derived from the hyperplastic glands of MT, MTK, and MMT mice. The epithelial lineage of the different cell lines was confirmed by expression of pan-cytokeratin, a marker of epithelial differentiation (data not shown). c-Src localization was determined by confocal microscopy. Immunofluorescent staining was used to compare the localization of c-Src in the MT, MMT, and MTK cells. Activated c-Src is normally localized at the cell membrane and cytoplasmically. A large proportion of the MTK cells exhibited perinuclear localization where c-Src formed a ring around the nucleus of the cell. Such a pattern was less observable in the MT and MMT cells (Figure 8b), suggesting that Muc1 may play a role in the localization of c-Src at the plasma membrane.

Discussion

We analysed MMTV-PyV MT mammary tumors on a Muc1 wild-type background compared with tumors of MMTV-PyV MT mice on a Muc1 null background to specifically delineate the effect of Muc1 on PyV MT-
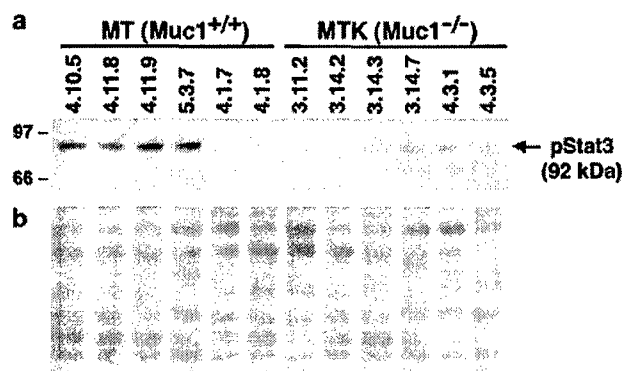

Figure 6 Hyperplastic glands from MT mice have higher levels of activated Stat3 than glands from MTK mice. (a) MT and MTK mice were analysed at 8 weeks of age. Lysates (100 μg) from MT and MTK mice were analysed by Western blot for activated Stat3 using an anti-phospho-stat3 (Y705) antibody. (b) Equal protein loading was determined using Ponceau S staining
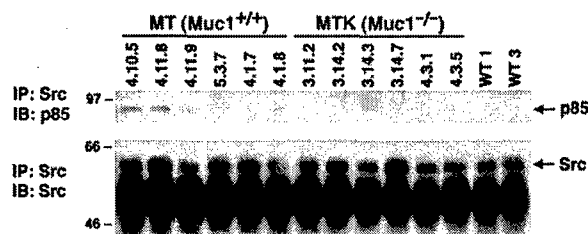

Figure 7 c-Src binds p85 only in the presence of Muc1. Hyperplastic glands from 8-week-old MT and MTK mice were immunoprecipitated with anti-Src (sc-18) and immunoblotted for p85 (upper panel) or for c-Src (sc-18) (lower panel)
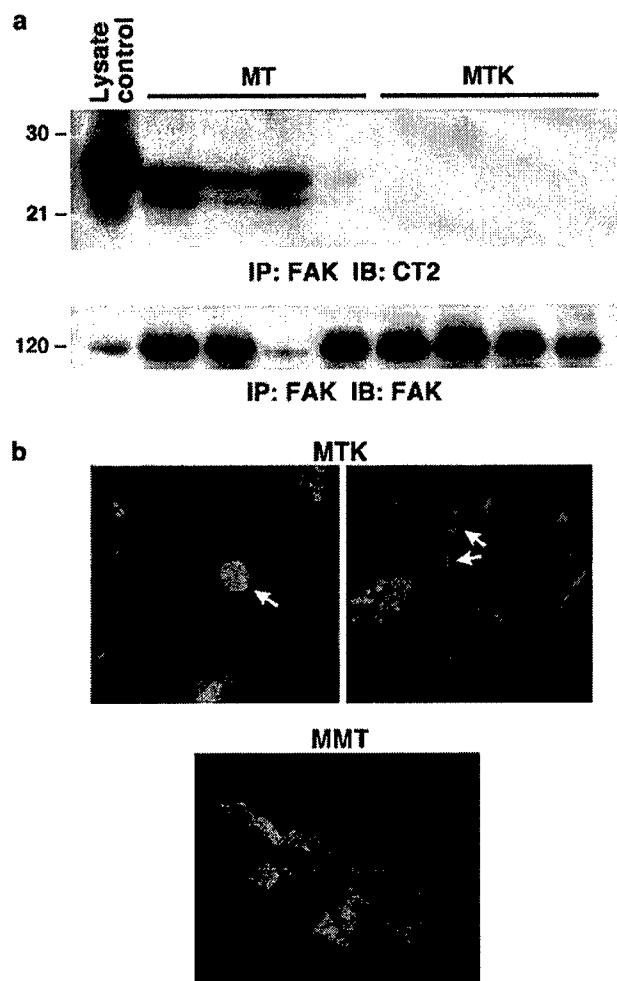
a

IP: FAK IB: CT2

IP: FAK IB: FAK

b

Figure 8 Muc1 interacts with FAK in tumors from MT mice and potentially influences c-Src cellular localization. (a) FAK was immunoprecipitated from tumor lysates (1 mg) from MT and MTK mice and immunoblotted for CT2 (upper panel) or for FAK (bottom panel). Tumor lysate (100 μg) from MT mice was used as positive control for Muc1 and FAK expression. (b) c-Src localization was determined by immunofluorescence. Primary cells from MTK and MMT mice were stained with anti-c-Src (2–17, red) and the nuclear TO-PRO 3 stain (blue). Sections were examined at × 250 magnification

induced mammary tumorigenesis. We identified an *in vivo* association between Muc1 and c-Src in PyV MT-induced tumors, which falls in line with previous *in vitro* studies and thus substantiates a physiological relevance for the association (Li *et al.*, 2001a). Src phosphorylates the cytoplasmic tail of MUC1 at the Y46 within the YEKV site, which constitutes a consensus SH2 binding site for Src (Li *et al.*, 2001a). The SH3 domain of Src has also been shown to be involved in mediating the interaction with MUC1 (Li *et al.*, 2001a). Interestingly, it has been demonstrated that the intracellular targeting of c-Src is a kinase-independent mechanism and rather relies on its SH3 domain with some modulation from the SH2 domain (Kaplan *et al.*, 1994). As such, the binding of c-Src to

MUC1 could serve to regulate the intracellular targeting of c-Src, which is critical to its functioning at the cell membrane.

We hypothesized that the influence of Muc1 on PyV MT-induced tumorigenesis could be to increase c-Src activity. We assessed the effect of Muc1 expression on c-Src activity by carrying out c-Src kinase assays on tumors from MMT, MT, and MTK mice and observed no significant effect of MUC1/Muc1 expression levels on c-Src activity. This result is perhaps not surprising, given previous results that showed that the associated kinase activities in mammary tumors of PyV MT mutants decoupled from either the Shc or the PI3K signaling pathways were comparable to that of wild-type PyV MT (Webster *et al.*, 1998). The failure to observe a significant difference in Src activity in the absence of Muc1 could simply result from the heterogeneity of the tumor samples and the elevated Src kinase activity in the tumors, whereby subtle changes in Src activity could pass undetected. Despite the high levels of expression of c-Src and Muc1 in the MT-induced tumors, only a fraction of both are associated, which could provide an explanation for the lack of detectable differences in c-Src activity. Alternately, these results could indicate that Muc1 influences PyV MT-induced tumorigenesis through an alternative mechanism, such as promoting the association of c-Src with its substrate proteins.

Examination of hyperplastic glands from MMTV-PyV MT glands on a Muc1 wild-type or Muc1 null background established that Muc1 does indeed influence the association of c-Src with known substrates. The presence of Muc1 in MT-induced tumors promoted the association between c-Src and β-catenin. Tyrosine phosphorylation of β-catenin by c-Src at Y654 has been shown to disrupt the binding of β-catenin to E-cadherin (Roura *et al.*, 1999). However, analysis of tumors from MT and MTK mice did not reveal a substantial change in the amount of β-catenin in association with E-cadherin, suggesting an alternate mechanism for weakening cadherin-dependent adhesions in PyV MT tumors.

We also observed increased levels of activated pStat3, a downstream target of c-Src activation, in the presence of Muc1. Muc1 may be enhancing the activation of Stat3, possibly by its association with both EGFR and c-Src, which have been shown to activate Stat3 (Olayioye *et al.*, 1999). Stat3 is involved in promoting cell cycle progression and cellular transformation and preventing apoptosis (Bromberg *et al.*, 1999; Catlett-Falcone *et al.*, 1999a, b; Turkson and Jove, 2000). Some PyV MT tumors that lack Muc1 exhibit a decrease in Erk2 activation (data not shown). Activation of MAPK in association with MUC1 has been demonstrated *in vitro* and in the mammary glands of MMTV-MUC1 transgenic mice (Meerzaman *et al.*, 2001; Schroeder *et al.*, 2001). In addition, mammary gland tumors induced by overexpression of MUC1 showed increased Erk activation, and involuting MMTV-MUC1 mammary glands expressed higher levels of activated Erk2 (Schroeder *et al.*, 2004). Erk2 has been reported to play a critical role in mediating proliferation in breast cancer cells (Flury *et al.*, 1997). Increases in Stat3 and Erk2

activity could provide a mechanism for increased progression of Muc1-expressing PyV MT tumors. Muc1 expression may be enhancing the activation of Stat3 and Erk2, possibly by its association with EGFR and c-Src. Interestingly, analysis of hyperplastic glands from MT and MTK mice suggested a decrease in EGFR tyrosine phosphorylation in the glands of MTK mice lacking Muc1 (data not shown).

Muc1 expression also stimulates the interaction between c-Src and p85 in the hyperplastic glands of MT mice. The interaction of PyV MT with p85 is a key event in PyV MT-induced transformation (Whitman et al., 1985; Courtneidge and Heber, 1987). Activation of PI3K signaling influences many aspects of cell behavior including proliferation, growth, survival, and cytoskeletal organization (Cantley, 2002). Mutation of the p85 binding site on PyV MT hindered its capacity to induce tumors in animals (Talmage et al., 1989; Freund et al., 1992). In a previously published study, transgenic mice expressing a mutated form of PyV MT decoupled from p85 binding developed mammary epithelial hyperplasias that were highly apoptotic, suggesting that PyV MT binding to p85 affected cell survival (Webster et al., 1998). The analysis of MT and MTK glands did not reveal changes in the activation of Akt, a downstream target of PI3K signaling affecting cell growth and cell survival. Also, hyperplasias and tumors from MT and MTK mice did not exhibit a difference in the rate of apoptosis, suggesting that this may not be the major pathway affected by the lack of Muc1 expression (unpublished data). In addition to influencing growth and survival, PI3K mediates activation of the Rac GTP binding protein, which plays a major role in remodeling the actin cytoskeleton (Rodriguez-Viciana et al., 1997). The p85 subunit of (PI3K) is also among the well-characterized c-Src interacting partners. The association between c-Src and the p85 subunit of PI3K is thought to modulate the localization of activated c-Src at the cell membrane (Fincham et al., 2000). As such, the presence of Muc1 may be influencing the localization of c-Src at the cell membrane through modulating its association with p85. This is supported by the translocation to the plasma membrane of c-Src in MT- and MMT-derived epithelial cells as compared to MTK.

Finally, we demonstrate a novel association between Muc1 and a known Src substrate, FAK, in the PyV MT tumors, which potentially promotes the peripheral targeting of activated c-Src. FAK is a nonreceptor protein-tyrosine kinase localized within focal adhesions where the cytoskeleton interacts with the extracellular matrix via integrin receptors. FAK plays an important role in integrin-mediated signaling and in modulating processes such as cell growth, differentiation, survival, and migration (Hanks et al., 2003). Following activation by c-Src phosphorylation, FAK interacts with a number of downstream signaling molecules including the adapter protein Grb2 and the p85 subunit of PI3K (Schlaepfer et al., 1999). The binding of MUC1 to Src can help localize the kinase at the cell periphery where it can be in close proximity to target molecules such as β-catenin, FAK, and p85. The interaction between MUC1 and FAK has not been described previously. Further investigation into the regulation of these associations will shed light on the role of MUC1, Src, and other proteins on invasion and metastases.

It has been established that Muc1 plays an important role in the progression of PyV MT-induced tumorigenesis. Our data provide insights into the possible mechanism for the significant delay in tumor progression that is observed in the absence of Muc1. We have shown that Muc1 levels have little effect on Src kinase activity. Instead, we suggest that the interaction of Muc1 with c-Src promotes its binding to downstream targets and influences its intracellular localization. Figure 9 represents a model for the possible ways Muc1 could influence c-Src signaling. As such, the ability of Muc1 to potentiate c-Src signaling could influence the proliferation and migration of tumor cells. Our understanding of the critical role of Muc1 signaling in PyV MT-induced oncogenesis will be invaluable in delineating the effects of MUC1 overexpression in human breast cancer.

Materials and methods

Transgenic mouse models

All studies were performed on the FVB strain of mice with either wild-type Muc1, transgenic human MUC1, or Muc1 knockout (described in Table 1). The generation of Muc1 knockout mice and the human MUC1 transgenics has been previously described (Spicer et al., 1995b; Rowse et al., 1998). The MUC1 transgenic mice, initially generated on the C57BL/6 background, were congenic with the FVB background, as were the Muc1$^{-/-}$ mice. To study the role of MUC1 in tumor formation, MMTV-PyV MT transgenic mice (a generous gift of Dr W Muller, McMaster University, Ontario) were crossed with Muc1 knockout mice or MUC1 transgenic mice.

Protein analysis

Thoracic mammary glands were prepared for protein analysis as previously described (Schroeder et al., 2004).

Antibodies

CT2 is a hamster monoclonal antibody developed in-house, which recognizes the cytoplasmic tail of both human MUC1 and mouse Muc1. HMFG-2 and B27.29 are mouse monoclonal antibodies that recognize the tandem repeat of human MUC1. HMFG-2 was a kind gift of Dr Joyce Taylor-Papadimitriou (ICRF, London, UK). B27.29 and FITC-B27.29 were kindly provided by Biomira (Edmonton, Alberta, Canada). c-Src (sc-18) from Santa Cruz was used for immunoprecipitation and immunoblotting. The monoclonal anti-Src (2–17) used for immunofluorescence was a kind gift from Dr Sarah Parsons (University of Virginia Health System, Charlottesville). Commercial antibodies used were β-catenin H-102 used for immunoprecipitation (10 μg per immunoprecipitation) and β-catenin C-18 used for immunoblotting (both from Santa Cruz), dpErk1/2 (Sigma), pStat3, p85, FAK, and c-Myc (Upstate Biotechnology), pAkt (Cell Signaling), and E-cadherin (Zymed).
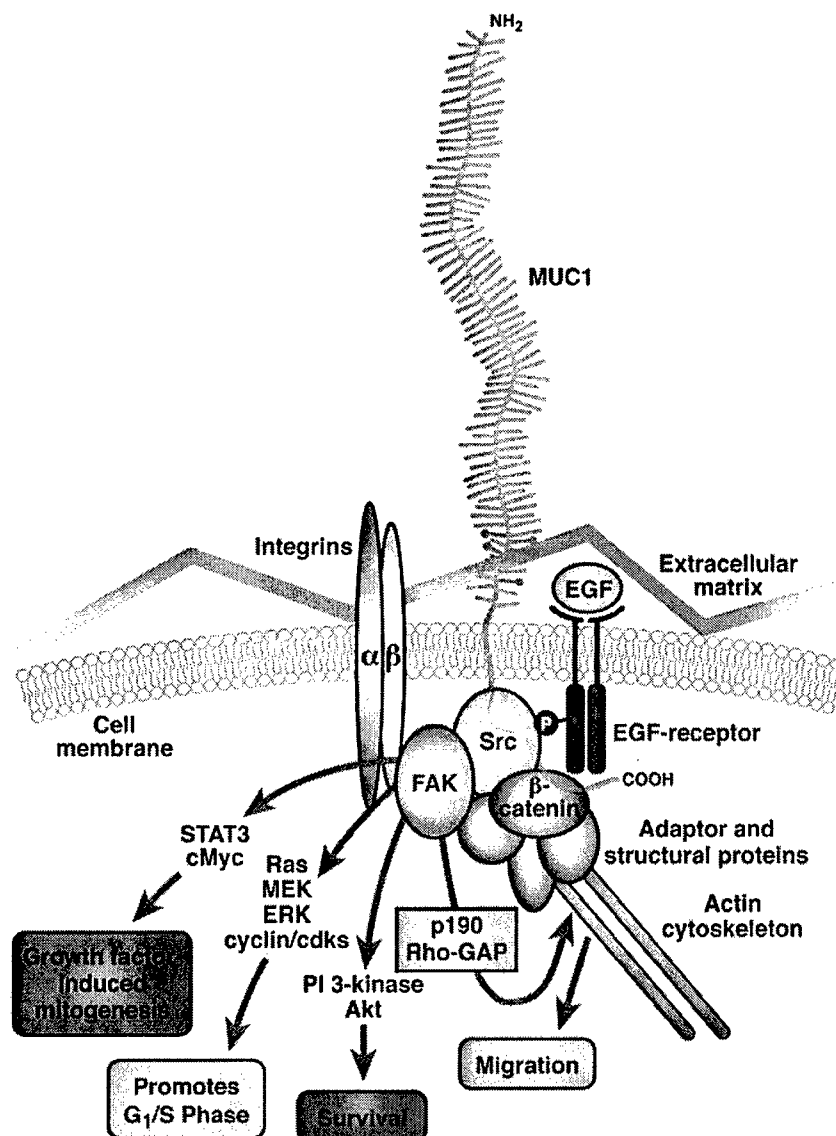
Figure 9 Model illustrating how Muc1 could be influencing c-Src signaling. We hypothesize that the overexpression of Muc1 by tumor cells may potentiate c-Src signaling by bringing it closer to its downstream targets such as β-catenin, FAK, and p85 at the cell membrane. Activation of c-Src influences various aspects of cell behavior including growth, proliferation, survival, and migration. This figure was adapted from MC Frame. (2002). Src in cancer: deregulation and consequences for cell behaviour. *Biochim. Biophys. Acta*, **1602**, 114–130, with permission from Elsevier

Immunofluorescence

Tissues were prepared for immunofluorescence as described previously (Schroeder *et al.*, 2004). The primary antibodies were used at the following dilutions: CT2 (1 : 200) and Src, 2–17 (1 : 1000). The nuclei were visualized using TO-PRO 3 from Molecular Probes (1 : 1000) and cytokeratin was stained using FITC-conjugated pan-cytokeratin antibody from Sigma used at 1 : 500.

In vitro kinase assay

Protein lysate (1 mg) from mammary gland tumors or normal mammary glands was used to immunoprecipitate Src as described above. An assay kit (Upstate Biotechnology) was used to measure Src kinase activity.

Conjugating CT2 to agarose beads

To avoid contamination with immunoglobulin heavy chain during immunoprecipitation, we used the Seize Primary Immunoprecipitation Kit (Pierce).

Generating primary cultures from mouse mammary glands

Primary mouse mammary epithelial cells were prepared from virgin mice. In brief, dissected mammary glands were minced with a razor blade, transferred to a plate containing 0.5 mM EDTA in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% glutamax, and 1% penicillin/streptomycin, and incubated on a shaker at 37°C for 1 h. The tissue was then filtered through a 40 μm nylon mesh. The filtrate contained dissociated cells, which were collected by centrifugation at 2000 r.p.m. for 2 min. The cells were washed

twice and resuspended in DMEM supplemented with 10% fetal calf serum, 1% glutamax, and 1% penicillin/streptomycin.

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MUC1 overexpression results in mammary gland tumorigenesis and prolonged alveolar differentiation

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MUC1 is a transmembrane mucin that was initially cloned from malignant mammary epithelial cells as a tumor antigen. More than 90% of human breast carcinomas overexpress MUC1. Numerous studies have demonstrated an interaction between MUC1 and other oncogenic proteins such as β-catenin, erbB receptors and c-Src, but a functional role for MUC1 in transformation has not been identified. We previously reported the development of transgenic mice that overexpress human MUC1 in the mouse mammary gland (MMTV-MUC1). Analysis of these transgenic mice at an early age demonstrated the ability of MUC1 to potentiate EGF-dependent activation of MAP kinase signaling pathways in the lactating mammary gland. We now report that multiparous MMTV-MUC1 transgenic mice stochastically develop unifocal mammary gland carcinomas late in life. Molecular analysis of these tumors shows a tumor-specific coimmunoprecipitation between MUC1 and β-catenin. Examination of the contralateral glands in MMTV-MUC1 transgenics demonstrates that the development of frank carcinomas is accompanied by a failure of multiparous glands to undergo postlactational involution. Furthermore, uniparous MMTV-MUC1 transgenic mice display decreased postlactational apoptosis, elevated whey acidic protein expression and aberrant pErk2 activation. These findings are the first to determine that MUC1 overexpression promotes in vivo transformation of the mammary gland.
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Introduction

MUC1 is a transmembrane mucin that was initially cloned from malignant mammary epithelial cells as a tumor antigen (Gendler et al., 1987). MUC1 contains both a heavily glycosylated extracellular domain and a highly conserved cytoplasmic domain (Gendler et al., 1990; Lan et al., 1990; Ligtenberg et al., 1990; Wreschner et al., 1990). While it is normally expressed on the apical surfaces of most glandular epithelia, MUC1 is expressed over the entire surface of transformed epithelial cells, displaying an altered pattern of glycosylation.

While MUC1 has been identified as aberrantly expressed in greater than 90% of human breast carcinomas (Zotter et al., 1988; Girling et al., 1989), the identification of a functional role for that overexpression has been elusive. Transgenic and knockout mice have provided insight into the role of MUC1 in tumor development, with the loss of Muc1 resulting in a reduction in tumor progression. Transgenic mice expressing the polyomavirus middle T antigen (PyV MT) under control of the mouse mammary tumor virus (MMTV) promoter exhibit a significant delay in tumor growth and a trend towards decreased metastasis when bred onto a Muc1 knockout background (Spicer et al., 1995b). Also, lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 model of breast cancer (Schroeder et al., 2003). Mammary tumors of MMTV-PyV MT and MMTV-Wnt-1 transgenic mice overexpress Muc1, and as such recapitulate the overexpression of human MUC1 observed in most breast carcinomas. Together, these studies indicate that MUC1 is not merely overexpressed in breast cancer but also plays an important role in the progression towards invasive carcinoma.

Numerous observations point to a functional role of MUC1 in signal transduction. The 72 amino-acid tail of MUC1 contains seven tyrosines, six of which are 100% conserved across mammalian species. The cytoplasmic tail of MUC1 contains potential docking sites for SH2-containing proteins in addition to a number of possible kinase recognition sites (Spicer et al., 1995a). Indeed, MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as epidermal growth factor receptor (EGFR), erbB2,

erbB3, erbB4, c-Src, protein kinase C delta (PKCδ), Grb2, β-catenin, GSK3β and p120^ctn (Pandey et al., 1995; Yamamoto et al., 1997; Li and Kufe, 2001; Li et al., 2001a, b; Schroeder et al., 2001, 2003; Ren et al., 2002). In vitro studies show that a defined sequence in the cytoplasmic tail of MUC1 (SXXXXXSSLS) interacts with β-catenin, and can compete with E-cadherin at adherens junctions for binding of β-catenin when expressed at high levels (Yamamoto et al., 1997). The serine/threonine kinase GSK3β can also bind the cytoplasmic domain of MUC1, and binding to this kinase competes away binding between MUC1 and β-catenin in breast cancer cell lines (Li et al., 1998). Studies in our lab determined that MUC1 and β-catenin biochemically interact in a tumor-specific manner in MMTV-Wnt-1 transgenic mice (Schroeder et al., 2003). Finally, both c-Src and the erbB receptor tyrosine kinases phosphorylate MUC1, as well as the serine/threonine kinase PKCδ, and these phosphorylation events promote interactions between β-catenin and MUC1 (Li et al., 2001a, b; Schroeder et al., 2001; Ren et al., 2002). These observations indicate that the cytoplasmic domain of MUC1 may provide a proto-oncogenic signal.

We have previously reported the generation of transgenic mouse models overexpressing various forms of MUC1 in the mouse mammary gland (Schroeder et al., 2001). Using the MMTV promoter, we directed the expression of either full-length, cytoplasmic domain-deleted or tandem repeat-deleted human MUC1 to the mammary gland in an effort to understand the effects of MUC1 overexpression in this tissue. These studies allowed us to determine a variety of signaling partners and downstream effects of MUC1 overexpression (including interacting with erbB receptors and potentiation of Erk1 and Erk2 activation). An expansion of our analysis of the MMTV-MUC1 transgenics included a long-term study of MUC1 overexpression, which resulted in the observation that MUC1 overexpression results in frank neoplasia accompanied by a failure of multiparous glands to dedifferentiate and involute. We have determined that this lack of involution can be observed in nontumor-bearing mammary glands as well, in that postlactational regression is suppressed by MUC1 overexpression. These data show, for the first time, that MUC1 can act as a promoter of transformation in the mammary gland. Furthermore, these data indicate that the mechanism for MUC1-induced tumorigenesis may be through the inhibition of normal glandular involution.

Results

MUC1 overexpression results in the stochastic formation of mammary gland tumors

We previously described the analysis of three MUC1 transgenic mouse models, one overexpressing full-length human MUC1 (MMTV-MUC1), a second overexpressing human MUC1 lacking the cytoplasmic domain

(MMTV-MUC1ΔCT) and a third overexpressing human MUC1 lacking the tandem repeat extracellular domain (MMTV-MUC1ΔTR) (Schroeder et al., 2001). The initial characterization of these transgenic mice focused on signal transduction initiated by MUC1 overexpression, resulting in the determination that MUC1 interacts with erbB receptors, and can modify the activation of Erk1/2 mitogen-activated protein kinases (MAPK) in an EGF-dependent manner. An expansion of our previous study included determining the effects of MUC1 overexpression over the lifetime of the mouse mammary gland. MMTV-MUC1 transgenic mice from two different founders (#9 and #15) were continuously bred for 12 months and then monitored as they aged. Cytoplasmic domain-deleted MUC1 transgenic mice (MMTV-MUC1ΔCT, #23) and wild-type controls were housed similarly. Tumors were observed in five out of eight MMTV-MUC1 mice and none were observed in the eight age-matched wild-type mice. As such, tumor incidence in the MMTV-MUC1 transgenic mice was 62% higher than in the wild-type mice ($P = 0.03$, 95% CI = 0.17–0.91). Statistical significance was determined using the Fisher's exact test. While primary mammary gland tumors were observed in both of the MMTV-MUC1 lines, no tumors were observed in either the MMTV-MUC1ΔCT transgenic line (three mice killed and analysed at 8 months and three mice at 20 months of age) or the wild-type control lines (analysed at 20 months). Pathological analysis of the tumors indicated a wide range of tumor subtypes in the MMTV-MUC1 transgenics, including papillary carcinoma (Figure 1a), solid carcinoma (Figure 1b), adenosquamous carcinoma (Figure 1c), microacinar carcinoma (Figure 1d) and lobular hyperplasia (Figure 1e) (M.D. Robert Cardiff, , U.C. Davis, personal communication).
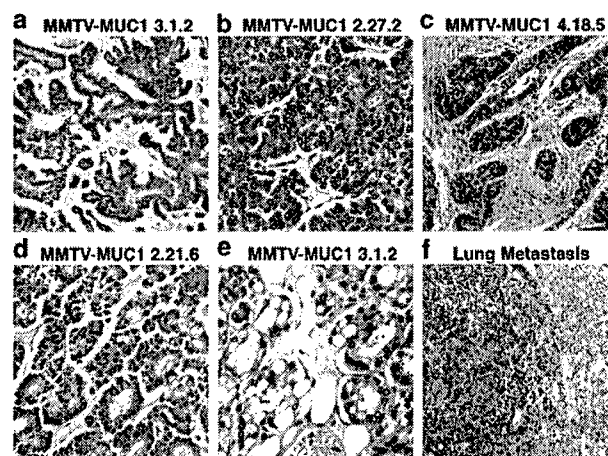

Figure 1 MUC1 overexpression results in the stochastic formation of mammary tumors. MMTV-MUC1 induced tumors, stained by hematoxylin and eosin, included a wide range of tumor subtypes including: papillary (a), solid carcinoma (b), adenosquamous carcinoma (c) microacinar carcinoma (d) and lobular hyperplasia (e). The expression of the human MUC1 transgene was detected in the lung metastasis of an MMTV-MUC1-induced tumor using an antibody to the FLAG epitope of the MUC1 transgene (f). Images (a–f) were captured at a magnification of × 200

In addition, we observed tumors in the lungs of four of five MMTV-MUC1 transgenic mice with mammary gland tumors. The lung tumors express the MUC1 transgene as determined by immunohistochemical detection of the FLAG epitope present in the transgenic construct (Figure 1f). While the FVB strain is prone to the development of primary lung tumors (at 14 months, 26% of FVB females develop spontaneous lung tumors; Mahler et al., 1996), the percentage of pulmonary tumor formation in the presence of the MMTV-MUC1 transgene was substantially higher. These data indicate that mice transgenic for MMTV-MUC1 can develop primary mammary gland carcinomas as well as pulmonary carcinomas.

MUC1 overexpression results in sustained differentiation in multiparous animals

All mice described above (whether tumor bearing or normal) were killed by 20 months of age and analysed for changes in the normal mammary gland architecture. The mammary glands of MMTV-MUC1ΔCT and wild-type females displayed the normal architecture expected from involutional regression (Figure 2c and d). However, contralateral mammary glands from the MMTV-MUC1 animals were not regressed, but instead maintained a fully differentiated phenotype, although none of the animals were pregnant at the time of being killed or had been pregnant for at least 4 months prior to being killed (Figure 2a and b). This ability to sustain a

differentiated phenotype suggested that MUC1 overexpression may either inhibit apoptotic regression that normally follows the end of lactation or may promote hyperproliferation of epithelial cells.

To determine the rate at which the transformed phenotype was observable in the mammary gland of transgenic animals, we isolated mammary glands from 8-month-old lactating MMTV-MUC1 transgenic animals and wild-type littermates. In tumor-free MMTV-MUC1 animals, mammary glands were analysed for changes in normal architecture. In the MMTV-MUC1 transgenics but not in the age-matched wild-type females, we observed regions of the mammary gland that appeared histologically atypical from the normal lactating gland (Figure 3a, c). Hyperplastic epithelial cells were surrounded by adipose tissue and some secretory droplets were present despite the absence of full lactational development in those areas of the gland. In addition, we analysed a multiparous MMTV-MUC1 mammary gland that was not pregnant or lactating at the time of being killed. Histological analysis of this particular MMTV-MUC1 mammary gland revealed hyperplasia and abnormal architecture (Figure 3b) (M.D. Thomas Lidner, Mayo Clinic Scottsdale, personal communication).

MUC1 and β-catenin interact in MMTV-MUC1 tumors and hyperplasia and in MMTV-MUC1 lactating glands

β-catenin interacts with the cytoplasmic tail of MUC1 in a tumor-specific manner (Schroeder et al., 2003). Through coimmunoprecipitation studies, we found that β-catenin interacts with the cytoplasmic tail of MUC1 in all MMTV-MUC1 tumors, but not in control wild-type mammary glands (Figure 4, upper panel). This association between MUC1 and β-catenin was also detected in the lactating glands of 8-month-old MMTV-MUC1 mice and in two out of four of the age-matched lactating wild-type mice (Figure 5b, upper panel). The interaction that occurs in the wild-type lactating gland could be attributed to the overexpression of Muc1 that occurs during lactation and the cellular changes associated with milk secretion. Note that while transgenic MUC1 was expressed (Figure 5a), overall levels of both β-catenin and endogenous Muc1 were similar in both the transgenic and wild-type mammary glands (Figure 5b, middle and

a   MMTV-MUC1   b   MMTV-MUC1

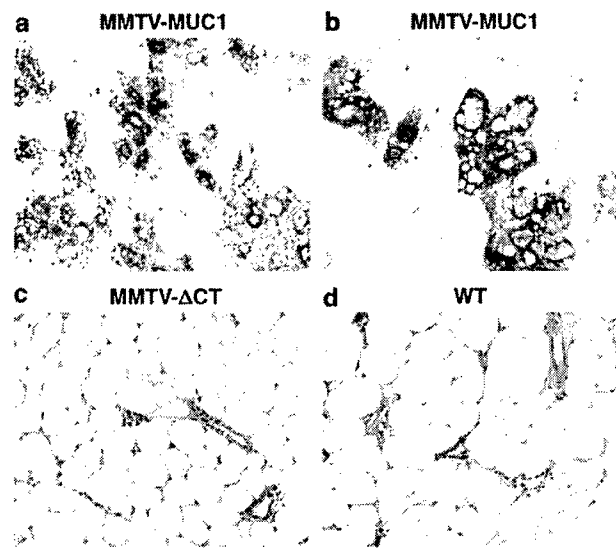c   MMTV-ΔCT   d   WT

Figure 2 MUC1 overexpression results in sustained differentiation in multiparous females. Glands of MMTV-MUC1 multiparous females that had not undergone a pregnancy in greater than 4 months retained the appearance of pregnancy, with fully differentiated glands and alveoli (a and b) as opposed to fully regressed glands in the MMTV-ΔCT (c) and wild-type females (d), both stained by hematoxylin and eosin. The hyperplastic glands of MMTV-MUC1 transgenics show expression of the transgene upon immunohistochemical analysis, using an antibody (B27.29) that recognizes human MUC1 but not mouse Muc1. Images were taken at a magnification of × 200

a   MMTV-MUC1 2.51.7 (L)   b   MMTV-MUC1 3.21.10 (V)   c   Lactating Wild-type
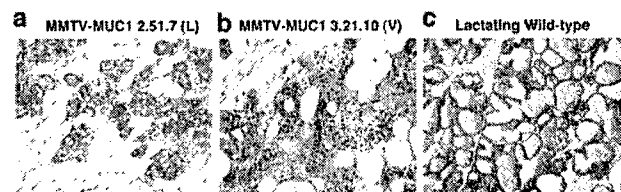
Figure 3 Mammary hyperplasia is evident in 8-month-old MMTV-MUC1 transgenic glands. The glands were stained by hematoxylin and eosin. Areas of hyperplasia were present in the 8-month-old MMTV-MUC1 lactating gland (a, (L) is for lactating) and similar areas of hyperplasia were observed in a nonpregnant and nonlactating gland of an 8-month-old MMTV-MUC1 female (b, (V) is for virgin). (c) is a lactating wildtype gland
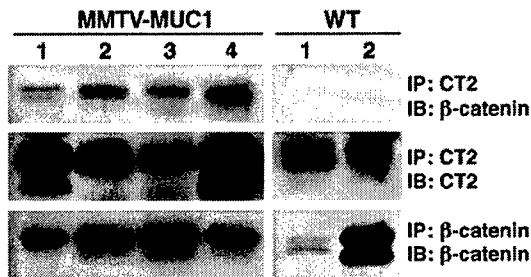
Figure 4 MUC1 associates with β-catenin in the MMTV-MUC1 tumors. Lysates (1 mg) of MMTV-MUC1 tumors and control wild-type (WT) mammary glands were immunoprecipitated with an anti-MUC1 antibody (CT2) and immunoblotted with anti-β-catenin, upper panel. The levels of MUC1 were determined by immoblotting with anti-MUC1 (CT2), middle panel. β-catenin was immunoprecipitated from normal and tumor lysates and detected by immunoblotting with anti-β-catenin, bottom panel. The level of β-catenin detected in WT2 appears high, which is apparently due to mouse-to-mouse variation

bottom panels). In addition, MUC1 was found to interact with β-catenin in a hyperplastic gland from a virgin 8-month-old nonpregnant nonlactating MMTV-MUC1 transgenic (Figure 5b, animal #3.21.10, upper panel).

Increased pErk1 and pErk2 activation in the MMTV-MUC1 mammary glands

Previous studies with the MMTV-MUC1 transgenics have demonstrated that high levels of MUC1 in the lactating mouse mammary gland strongly induced EGF-dependent activation of the extracellular signal-regulated protein kinases Erk1 (p44) and Erk2 (p42) (also known as MAPKs) (Schroeder *et al.*, 2001). Erk1 and Erk2 are two downstream effectors of the EGFR and subsequent Ras signaling pathway, and are commonly activated in EGFR-induced transformation (Grant *et al.*, 2002). Thus, we were interested in determining the effects of MUC1 overexpression on Erk1 and Erk2 activation. We analysed seven approximately 8-month-old transgenic and six wild-type glands, and observed increased levels of dual phosphorylated Erk1 and Erk2 in five out of seven of the MMTV-MUC1 lactating glands as well as in the hyperplastic MMTV-MUC1 3.21.10 gland, while total Erk levels were similar to wild-type glands. A representative image is shown in Figure 5c.

MMTV-MUC1 transgenics exhibit delayed postlactational involution

In an effort to understand the mechanism of prolonged glandular differentiation in the older, multiparous MMTV-MUC1 transgenics, we analysed young uniparous females for differences in postlactational involution. Following lactation, the mammary gland normally involutes via apoptosis, resulting in a defined series of gene transcriptional events, adipose tissue deposition and the loss of differentiated epithelial alveoli (Strange *et al.*, 2001). MMTV-MUC1 transgenic and wild-type females were allowed to lactate for 6–10 days followed
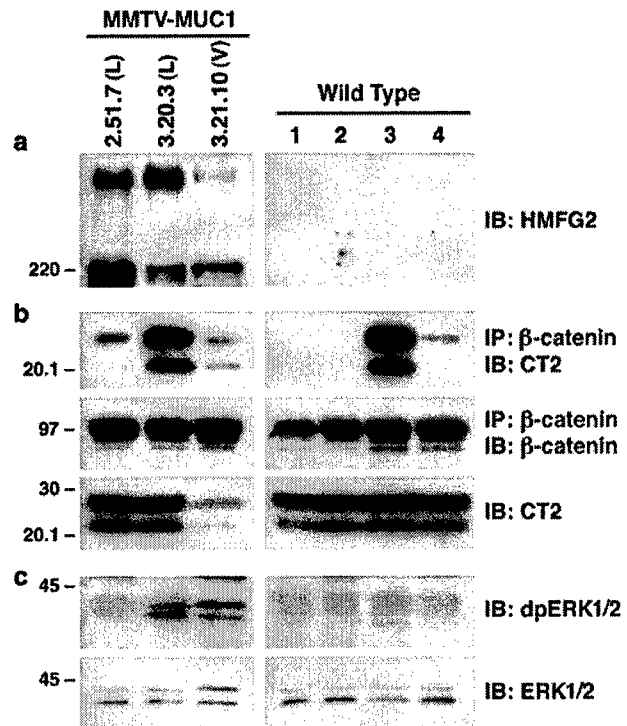

Figure 5 MUC1 associates with β-catenin in MMTV-MUC1 glands and increased Erk1/2 activity correlates with MUC1 overexpression. Mammary gland lysates (100 μg) of lactating (L) 8-month-old MMTV-MUC1 transgenic and wild-type mice were immunoblotted for the transgene using HMFG2 (a). MMTV-MUC1 3.21.10 (V) is a hyperplastic gland of a nonlactating and nonpregnant MUC1 transgenic. Lysates (1 mg) of 8-month-old transgenic and wild-type females were immunoprecipitated with anti-β-catenin and immunoblotted with CT2, an anti-MUC1 antibody (b, upper panel). The levels of β-catenin were determined by immunoblotting using anti-β-catenin antibody (Santa Cruz Biotechnology) (b, middle panel). CT2 detects the expression levels of both MUC1 transgene and endogenous Muc1 (b, bottom panel). Mammary gland lysates (100 μg) of 8-month-old MMTV-MUC1 transgenic and wild-type mice were immunoblotted for dpErk1/2 (c, upper panel) and Erk1/2 (c, lower panel)

by pup removal to initiate involution. The animals were then killed at various time points and their mammary glands were analysed at days 2, 4, 6 and 8 of involution. We performed whole-mount analysis to assess any gross morphological differences between glands of MMTV-MUC1 transgenic and wild-type mice, and observed a delay in the postlactational involution of MMTV-MUC1 glands in comparison to the wild-type controls (Figure 6). At day 4 of involution, we noted the presence of lobuloalveolar structures in the mammary glands of both the MMTV-MUC1 transgenics (Figure 6a) and the wild-type controls (Figure 6d). By day 6 the collapse of lobulo-alveolar structures proceeded normally in the mammary glands of the wild-type females (Figure 6e). In contrast, the mammary glands of MMTV-MUC1 transgenics were still filled with lobuloalveolar structures (Figure 6b). At day 8 of involution, the wild-type glands had fully regressed (Figure 6f), while large,
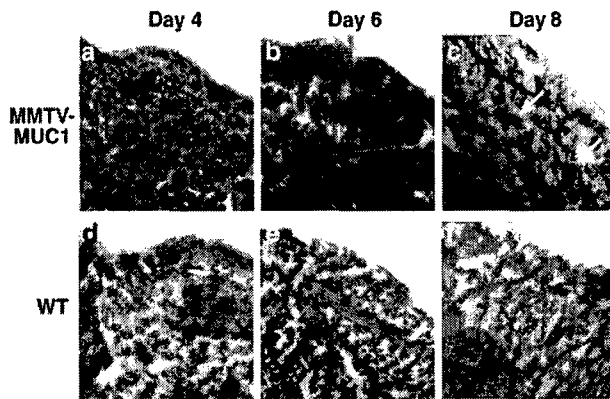
Figure 6 Mammary gland involution is delayed in the MUC1 transgenic mice. Representative images of whole mounts of involuting thoracic mammary glands from MMTV-MUC1 transgenic females (a–c) and wild-type controls (d–f) at day 4 of involution (**a** and **d**), day 6 of involution (**b** and **e**) and day 8 of involution (**c** and **f**). Arrows depict the large secretory ducts that are present in the MMTV-MUC1 glands at day 8 of involution. Images were captured at a magnification of ×250

Figure 7 MUC1 is highly expressed within the alveolar lumens and secretory ducts of MUC1 transgenic mice. Paraffin-embedded inguinal glands from MUC1 transgenic females (a–c) and wild-type controls (d–f) at day 4 of involution (**a** and **d**), day 6 of involution (**b** and **e**) and day 8 of involution (**c** and **f**). Immunohistochemical analysis of MUC1 transgenic mammary glands (a–c) using anti-MUC1 B27.29 (recognizes the PDTRPAP epitope in the tandem repeat of human MUC1). Wild-type glands (d–f) stained with CT2 (recognizes the last 17 amino acids of the cytoplasmic tail of both human and mouse Muc1). Wild-type glands stained with B27.29 were negative (data not shown). Images were captured at a magnification of ×100

secretory ducts were still present in the MMTV-MUC1 glands (Figure 6c).

MMTV-MUC1 and control wild-type mammary glands obtained at days 4, 6 and 8 were also sectioned and stained with hematoxylin and eosin to examine gland differentiation histologically. We observed no significant differences between MMTV-MUC1 and wild-type glands at days 4 and 6. However, at day 8 MMTV-MUC1 transgenics, showed large secretory ducts that were no longer present in the wild-type controls (data not shown). This delayed involution at day 8 in the transgenic gland correlates with high expression levels of the MUC1 transgene within the large secretory ducts (Figure 7). We performed immunohistochemical analysis to examine MUC1 expression in MMTV-MUC1 and control wild-type glands at days 4, 6 and 8. Wild-type animals were stained for endogenous Muc1 (Figure 7d–f) and MMTV-MUC1 animals were stained for the expression of the MUC1 transgene using an antibody specific to human MUC1 (Figure 7a–c). Similar to wild-type Muc1, the MUC1 transgene was predominantly apically expressed within the alveolar lumens and secretory ducts. Figure 7f depicts the collapse of the lumenal spaces in the wild-type at day 8, while large lumenal spaces with appreciable amounts of MUC1 were present in the MMTV-MUC1 transgenics (Figure 7c).

Increased whey acidic protein (WAP) expression

WAP is normally highly expressed during lactation and its expression is downregulated during the early phase of involution (Burdon *et al.*, 1991; Marti *et al.*, 1999). We examined WAP expression by Western blot analysis in the transgenic and wild-type animals at days 2, 4, 6 and 8 of involution. As expected, WAP expression decreased in the wild-type mice from days 2 to 8. However, at day 6, a subset of MMTV-MUC1 glands (2/5) continued to
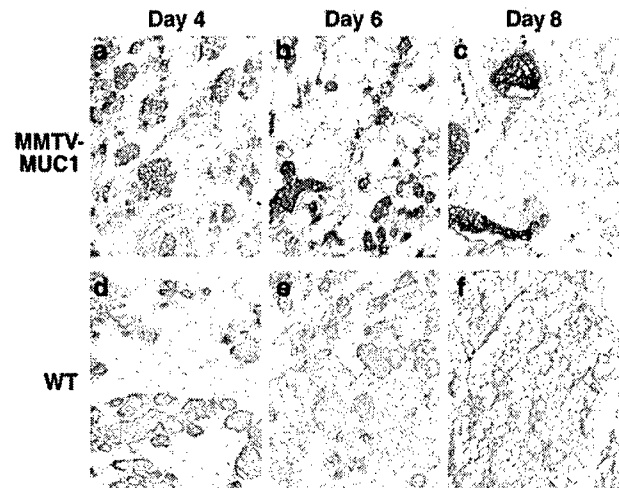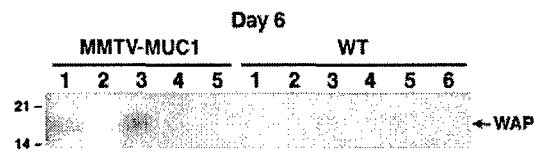

Figure 8 Increased WAP expression is observed in the mammary glands of MUC1 transgenics at day 6 of involution. Protein lysates (100 µg) from thoracic mammary glands of transgenic glands (MMTV-MUC1) and wild-type glands at day 6 of involution were examined by Western blot analysis for WAP expression. Increased WAP expression was observed in two out of five MUC1 transgenics, while it was completely absent in their wild-type counterparts at day 6 of involution

express elevated levels of WAP when compared to wild-type mice (Figure 8). We studied the expression levels and activation of a number of key apoptotic and antiapoptotic markers at days 2, 4, 6 and 8 of involution. We saw no observable differences in pStat3, pStat5, pAkt and caspase-3 (data not shown).

Decreased apoptosis in the MMTV-MUC1 mammary glands

Apoptotic cell death accounts for the disappearance of the epithelial compartment during mammary gland involution. In order to determine the mechanism by which MUC1 overexpression resulted in delayed mammary involution, we performed TUNEL assays to quantitate differences in apoptosis at day 2 of involution. We chose to analyse the mammary glands at that time
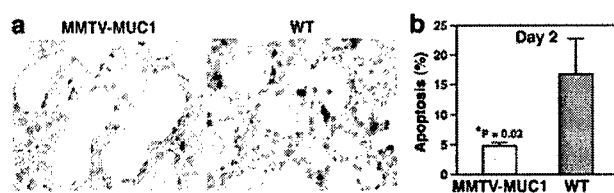
Figure 9 MMTV-MUC1 glands exhibit decreased levels of apoptosis at day 2 of involution. (a) Representative images of MMTV-MUC1 and wild-type glands at day 2 of involution analysed by the TUNEL assay. Brown staining depicts apoptotic nuclei. Images were captured at a magnification of ×400. (b) MMTV-MUC1 glands have a decreased percentage of apoptotic cells compared with wild-type glands at day 2 of involution
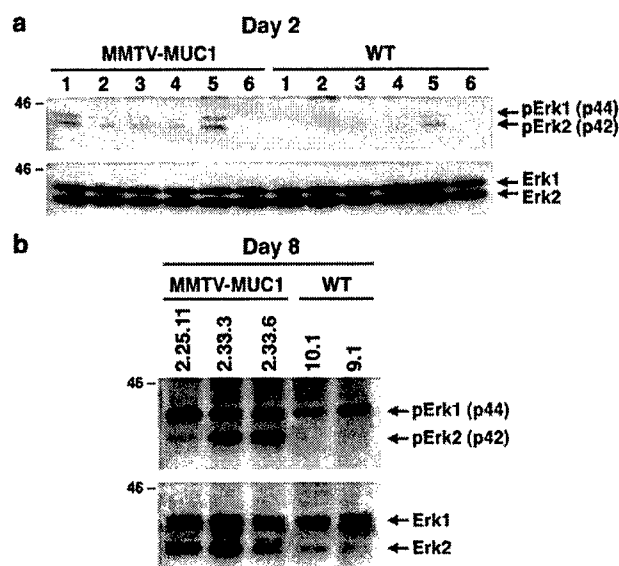

Figure 10 Erk1 and Erk2 activation is increased at day 2 of involution in MMTV-MUC1 glands and Erk2 activation persists at day 8 in MMTV-MUC1 transgenic mammary glands. Protein lysates (100 μg) from thoracic mammary glands of transgenic glands (MMTV-MUC1) and wild-type glands were examined by Western blot analysis for Erk1 and Erk2 expression and activation at day 2 (a) and day 8 of involution (b). Erk1/2 were detected with p42/44 (Cell Signaling) and antibodies specific to dual phosphorylated Erk1/2 were used to detect active Erks (Sigma). (a) Increased Erk1/2 activation is observed in MMTV-MUC1 glands at day 2 of involution (a, upper panel) and no differences were observed in the levels of Erk1/2 (bottom panel). (b) pErk2 is still present in the MMTV-MUC1 transgenic mammary glands at day 8 of involution, while pErk2 is absent in the wild-type counterparts (upper panel). There is corresponding decrease in Erk2 levels in the wild-type compared to MUC1 transgenics (lower panel)

point since that is when the majority of apoptosis takes place (Walker *et al.*, 1989). Figure 9a shows representative examples of TUNEL analysis on paraffin-embedded mammary glands of transgenic and wild-type mice. Mammary glands of MMTV-MUC1 transgenic mice displayed a reduced rate of apoptosis compared to the wild-type glands. We found that the percentage of apoptotic cells was 16.7 ($\pm$6.1) for the wild-type glands and 4.8 ($\pm$0.5) for MMTV-MUC1 transgenics, $P = 0.03$ from the two-sample *t*-test (Figure 9b).

Increased pErk2 activation in the MUC1 transgenic mammary glands

The MMTV-MUC1 transgenics display morphological inhibition of postlactational involution as detected by whole mount and histology (Figures 6 and 7), biochemical inhibition as determined by continued expression of the differentiation protein WAP (Figure 8) and are functionally inhibited as shown by the failure of MMTV-MUC1 glands to undergo wild-type levels of apoptosis (Figure 9). We next investigated if Erk1/2 was also aberrantly activated during involution. We examined mammary glands from both MUC1 transgenic and wild-type controls for their expression profile of Erk1 and Erk2 by Western blot analysis at days 2, 4, 6 and 8 of involution. While wild-type and transgenic glands displayed no difference in Erk1 and Erk2 activation at days 4 and 6 (data not shown), we detected increased Erk1/2 phosphorylation in the MMTV-MUC1 mammary glands when compared to their wild-type counterparts at day 2 (Figure 10a). In addition, there was increased Erk2 activation in the MMTV-MUC1 transgenics at day 8 of involution, compared to the wild-type glands (Figure 10b). This similarity between histological and biochemical alterations in the postlactational gland and glands contralateral to tumors may indicate that these alterations are requisite precursors to MUC1-induced transformation of the mammary gland.

Discussion

We have found that prolonged expression of MUC1 in the mouse mammary gland results in the stochastic

development of unifocal tumors. Coincident with the development of frank neoplasia, prolonged expression of MUC1 inhibited postlactational involution by the inhibition of apoptosis. This is the first report to demonstrate that increased MUC1 expression is not merely a byproduct of transformation, but can itself promote transformation in a physiological context.

Frequently, these same mechanisms are observed as normal epithelium progresses to transformation, as is observed in the formation of hyperplastic alveolar nodules in precancerous mammary glands (Strange *et al.*, 2001). Mammary gland involution is a developmentally regulated process triggered upon cessation of lactation and consists of elimination of unneeded epithelial cells by apoptosis and subsequent tissue remodeling. It has been demonstrated that premalignant hyperplasias do not respond to apoptotic stimuli and fail to regress during postlactational involution (Strange *et al.*, 2001). It is likely that the sustained differentiation and the delay in involution in the MMTV-MUC1 mammary glands resulted in the persistence of a population of epithelial cells that are resistant to apoptosis and hormonal regulation. In turn, these cells could acquire additional mutations leading to transformation and ultimately the development of mammary tumors.

In our study, we observed elevated Erk activation in lactating MMTV-MUC1 glands and in an MMTV-MUC1 hyperplastic nonlactating gland. Consistent with our findings, previous studies have linked MUC1 to Erk1/2 activation. We have previously demonstrated that MMTV-MUC1 transgenic mice undergoing lactation have the ability to activate Erk1/2 in response to EGF, whereas wild-type mammary glands do not (Schroeder et al., 2001). In a separate study, Meerzaman et al. (2001) demonstrated the ability of CD8/MUC1 chimeric receptors to activate Erk2 phosphorylation in COS cells. Erk1 and Erk2 are serine/threonine protein kinases that belong to the highly conserved family of MAPKs that function in mediating cellular responses to a variety of extracellular signals (Kolch, 2000). Overexpression of Erk1 and Erk2 has been detected in breast cancer cells with a five- to 10-fold increase in activity over benign conditions. The overexpression of Erk1 and Erk2 was detected in epithelial cells of primary breast cancer as well as at distant metastases (Sivaraman et al., 1997). A recent study found that Erk1 and Erk2 are not only overexpressed but also hyperactivated in human breast tumors compared with normal tissue. Interestingly, Erk2 expression levels were higher than Erk1 in most tumor samples (Mueller et al., 2000). Not much is known about the independent functions of Erk2 as opposed to Erk1; however, it has been reported that Erk2 plays a critical role in mediating EGF-stimulated proliferation in MCF-7 breast cancer cells (Flury et al., 1997). The specific downstream effects of Erk1 and Erk2 activation in the mammary epithelial cells are still not defined. However, a recent report indicates that inhibition of Erk1 and Erk2 activation results in increased apoptosis after BRCA1 expression in MCF-7 breast cancer cells (Yan et al., 2002). This suggests that the increases in Erk1 and Erk2 activation associated with MUC1 overexpression could provide a mechanism for the failure to eliminate cells by apoptosis, resulting in premalignant hyperplasia that contributes to mammary tumors.

The current question pertaining to understanding the role of MUC1 in cancer is delineating which domain of MUC1 is necessary to drive tumorigenesis. Much research has focused on studying the cytoplasmic domain of MUC1, which contains seven conserved tyrosine residues and a number of consensus binding motifs. MUC1 cytoplasmic tail interacts with a variety of proteins involved in neoplasia and cell adhesion such as EGFR, erbB2, erbB3, erbB4, c-Src, PKCδ, Grb2, β-catenin, GSK3β and p120ctn (Pandey et al., 1995; Yamamoto et al., 1997; Li and Kufe, 2001; Li et al., 2001a, b; Schroeder et al., 2001, 2003; Ren et al., 2002). c-Src and erbB receptor tyrosine kinases and the serine/threonine kinase PKCδ are among the kinases that are known to phosphorylate the cytoplasmic domain of MUC1. The phosphorylation of the cytoplasmic tail of MUC1 is thought to regulate its association with two antagonistic proteins, β-catenin and GSK3β. MUC1, through its various cytoplasmic tail interactions, may act as a scaffolding protein to enhance growth-promoting and oncogenic signals. It will be interesting to determine whether the lack of the cytoplasmic tail of MUC1 will alter the delayed involution phenotype observed in the MMTV-MUC1 mice.

Previously, our lab has demonstrated that tumor-specific MUC1/β-catenin complexes are detected in primary breast tumors and metastatic lesions (Schroeder et al., 2003). We speculated that these complexes may promote metastatic invasion by directing β-catenin to the leading edge of the cell and facilitating its interaction with fascin. Recently published studies described the association between the cytoplasmic tail of MUC1 and β-catenin in the nucleus of human pancreatic cancer cell lines and in the nucleus of multiple myeloma cells (Li et al., 2003a; Wen et al., 2003). In a recently published report, stable expression of human MUC1 in rat 3Y1 fibroblasts resulted in anchorage-independent growth of the cells and tumor formation upon injection into nude mice (Li et al., 2003b). Analysis of the tumor sections depicted the presence of the cytoplasmic tail of MUC1 in the nucleus in a complex with β-catenin (Li et al., 2003b). In fact, the cytoplasmic tail has been shown to activate transcription of cyclin D1, a β-catenin target gene. Mutation of Y46, a critical residue within the cytoplasmic tail of MUC1 that regulates its association with β-catenin, was found to attenuate the function of MUC1 as a transcriptional activator of Wnt target genes and also abrogate its transforming capability (Huang et al., 2003). In the current study, we have shown that the association between β-catenin and MUC1 exists in the MMTV-MUC1 induced tumors but not in the normal mammary gland. We also observe this association in the hyperplastic glands of the MMTV-MUC1 transgenic mice. This suggests that MUC1/β-catenin complex formation may be an early event in mammary transformation.

MUC1 has long been recognized as a tumor-associated antigen. More than 90% of human breast cancers overexpress MUC1. MUC1 overexpression in cancer is thought to influence adhesion, invasion and immune surveillance. Recent work has highlighted a role for the cytoplasmic domain of MUC1 in cell signaling that contributes to malignant transformation of epithelial cells. Similar to the human breast tumors, mouse models of breast cancer such as the MMTV-PyV MT and MMTV-Wnt-1 transgenic mice exhibit overexpression of Muc1. The lack of Muc1 expression causes a significant delay in tumor onset in the MMTV-Wnt-1 mice, suggesting that Muc1 is involved in the initiation stages of cancer in that β-catenin-dependent model. Recent in vitro studies demonstrate the ability of MUC1 to transform cells. We now show that the overexpression of MUC1 is directly involved in the in vivo transformation of the mammary gland.

Materials and methods

Transgenic animals

Transgenic mice that express the 42 tandem repeat human MUC1 cDNA (referred to as MMTV-MUC1) or human MUC1 lacking the cytoplasmic tail (referred to as MMTV-MUC1ΔCT) have been described previously (Schroeder et al., 2001). Transgenic mice were maintained on an inbred FVB

background and wild-type littermates were used as controls. Two separate MMTV-MUC1 lines (MMTV-MUC1#9 and #15), line #23 of MMTV-MUC1ΔCT and wild-type littermates were bred continuously for 12 months and then monitored for tumor development. Human MUC1 and mouse Muc1 are distinguished as such.

Induction of involution

Uniparous wild-type or transgenic MMTV-MUC1 female mice were allowed to lactate for 6–10 days to allow for full development of lactation, and the day the pups were removed was counted as day 0 of involution. Only mice having litters of >5 pups were included in the study. The animals were killed at 2, 4, 6 or 8 days after pup removal and the mammary glands were harvested.

Whole mounts

Whole mounts of thoracic and inguinal glands were prepared by spreading the glands on a glass slide and fixing in a 1:3 solution of glacial acetic acid and absolute ethanol for 1 h. Following fixation, the glands were washed with 95% ethanol for 15 min, followed by a wash with absolute ethanol for 15 min. The glands were defatted overnight through four changes of acetone. The glands were then rehydrated and stained overnight in 0.2% carmine and 0.5% aluminum potassium sulfate. Destaining was performed by washing for 15 min each in 70% ethanol then 95% ethanol followed by absolute ethanol and then 95% ethanol and finally 70% ethanol. The slides were then stored in glycerol.

Protein analysis

Thoracic glands were prepared for Western blot analysis as described previously in Schroeder *et al.* (2001). Protein concentrations were determined by BCA (Pierce). Protein lysates (100 μg) were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane (Immobilon). Immunoprecipitations were performed with 1 mg of protein lysate using recombinant protein G–agarose conjugate (Invitrogen). Antibodies for immunoblotting were obtained from the following sources: CT2, a hamster monoclonal antibody developed in house to the cytoplasmic tail of MUC1. CT2 recognizes the cytoplasmic tail of both human MUC1 and mouse Muc1. HMFG-2 and B27.29 are mouse monoclonal antibodies that recognize the tandem repeat of human MUC1. HMFG-2 was the kind gift from Dr Joyce Taylor-Papadimitriou (ICRF, London, UK). B27.29 was kindly provided by

Biomira (Edmonton, Alberta, Canada). β-catenin H-102 (10 μg) was used for immunoprecipitation and β-catenin C-18 was used for immunoblotting, both from Santa Cruz; Erk1/2 (Cell Signaling, #9102), dpErk1/2 (Sigma Chemical Company, M8159) and WAP (Santa Cruz Biotechnology, M16) were also used. For immunoblotting, the antibodies were used at the following dilutions: CT2 (1:500), HMFG-2 (1:20), B27.29 (1:1000), β-catenin (1:1000), Erk1/2 (1:1000), dpErk1/2 (1:10 000) and WAP (1:500). HRP-conjugated secondary antibodies were from Pierce and Jackson Laboratories and used at 1:25 000.

Immunohistochemistry

Inguinal mammary glands were fixed in methacarn (methanol:chloroform:acetic acid, 6:3:1) or formalin and processed as described previously (Schroeder *et al.*, 2001). Dilutions for antibodies are as follows. anti-MUC1, B27.29 (1:500), anti-Muc1 or anti-MUC1, CT2 (1:400). Lung metastases were stained with an antibody to the FLAG epitope tag, M5 (1:100, Sigma).

TUNEL

The Apoptag Plus Peroxidase *In Situ* Apoptosis Detection kit (Serologicals Corporation) was used to identify apoptotic nuclei within the mammary glands. Formalin-fixed inguinal mammary glands (at day 2 of involution) from three separate MMTV-MUC1 and three wild-type controls were analysed. The protocol outlined by the manufacturer was modified as described by Garrity *et al.* (2003). We stained duplicate sections and a minimum of 1000 cells was counted per section covering 20 randomly chosen fields ($\times 400$). The number of apoptotic cells was calculated as a percentage of total cell count. Statistical significance was determined using the two-sample *t*-test.

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